Investigation of Contaminants with Immunomodulatory Activity in Coagulation Factor Concentrates

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DECLARATION

In accordance with the University of Edinburgh regulations I declare that this thesis was composed solely by myself and the work reported is my own.

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ABSTRACT

It is now recognised that coagulation factor concentrates may cause immunological abnormalities in haemophilia patients, irrespective of HIV status. It has been proposed that these disturbances of the immune system are caused by either the massive protein load, specific impurities or by viruses in coagulation factor concentrates. There has been a recent claim that the major contaminant in factor VIII concentrates is transforming growth factor β (TGF-β) and its presence may partially explain the reported immunomodulatory effects in HIV negative haemophiliacs.

This study investigated the effects that coagulation factor concentrates of varying purity have on *in vitro* assays of immune function. The nature of the contaminants causing these effects was also investigated.

The results demonstrate that all conventional and ion-exchange chromatographically purified coagulation factor concentrates inhibit lymphocyte proliferation *in vitro*. These concentrates also contain measurable amounts of active TGF-β. In contrast, immunoaffinity purified and recombinant coagulation factor concentrates do not inhibit lymphocyte proliferation and contain very little active TGF-β. The levels of TGF-β detected in coagulation factor concentrates correlated with the degree of inhibition of lymphocyte proliferation. However, the amount of TGF-β present in the concentrates could account for only a small portion of the inhibitory activity. In addition, neutralising antibody specific to TGF-β only partially reversed the inhibitory effects that concentrates had on lymphocyte proliferation. In conclusion, while TGF-β is present in concentrates, the amount detectable (and its relative potency) suggest it is only a minor immunosuppressive contaminant in coagulation factor concentrates.

Part of the inhibitory activity of some higher purity products was removed by dialysis, suggesting that low molecular weight components, such as citrate were responsible for a proportion of the inhibitory activity of these products. No portion of the inhibitory activity of conventionally fractionated products was dialysable.

Gel filtration experiments on an ion-exchange purified factor VIII concentrate suggest the presence of several inhibitory components which appear variably in different batches of this concentrate. Six peaks of lymphocyte proliferation inhibitory activity were detected, one with a molecular weight of >970 kDa, three peaks of inhibitory activity with molecular weights of approximately 550 kDa, 220 kDa, 120 kDa, a peak with a molecular weight <68 kDa and one with a molecular weight of approximately 6.5 kDa. The inhibitory fractions with molecular weight <68 kDa contained active TGF-β and the fractions with a molecular weight of approximately 6.5 kDa were confirmed to be low molecular weight components used in the formulation of this product. The four high molecular weight components were not identified.

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ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
AET	aminoethylisothiouronium bromide
Ala	alanine
APC	activated protein C
Arg	arginine
bp	base pair
BPL	BioProducts Laboratory
BSA	bovine serum albumin
CD	cluster determinant
CHO	Chinese hamster ovary
ConA	concanavalin A
cpm	counts per minute
DEAE	diethylaminoethyl
DNCB	dinitrochlorobenzene
DTT	dithiothreitol
DDAVP	1-Deamino-8-D-arganine vasopressin (desmopressin)
E.C.A.C.C.	European Collection of Animal Cell Cultures
EDTA	eythylenediaminetetraacetic acid
EGF	epidermal growth factor
FCS	foetal calf serum
HIV	human immunodeficiency virus
HLA	human leukocyte anitgen
HMWK	high molecular weight kallikrein
HPVIII	High Potency Factor VIII
HRP	horse radish peroxidase
Ig	immunoglobulin
IL	interleukin
IFN	interferon
ITSS	insulin-transferrin-sodium selenite
kb	kilobase

LDCF	leukocyte derived chemotactic factor
LFA	leukocyte function associated antigen
LIF	leukocyte inhibitory factor
N.I.B.S.C.	National Instituite for Biological Standards and Control
NK	natural killer cell
OSD	organic solvent detergent
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PL	phospholipids
PMA	phorbol-myristate acetate
PTC`	plasma thromboplastin component
PTT	partial thromboplastin time
RFLP	restriction fragment length polymorphism
RID	radial immunodiffusion
S.A.P.U.	Scottish Antibody Production Unit
Ser	serine
SD	solvent detergent
SDS	sodium dodecyl sulphate
S.N.B.T.S.	Scottish National Blood Transfusion Service
SRBC	sheep red blood corpuscles
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	transforming growth factor beta
TNBP	tri (n-butyl) phosphate
Tyr	tyrosine
Val	valine
Vo	void volume
VNTR	variable number of tandem repeats
vWF	von Willebrand factor

CHAPTER 1

Introduction and Background

Factor VIII and factor IX play important roles in the blood coagulation cascade, Figure 1. The clinical importance of factor VIII and factor IX, in the maintenance of haemostasis is evident from the occurrence of haemophilia A and haemophilia B in individuals with reduced function or amount of these proteins.

1. The History of Haemophilia and its Treatment

Haemophilia is an ancient disease, first described as a severe haemorrhagic tendency found in males of certain families, by the Talmudic Rabbi Judah in 500 AD (Rosner, 1969). Although the existence of haemophilia was recognised as early as this the first widely read report in modern medical literature of a haemophilia-like condition was that by an American physician John Otto in 1803. Otto described the syndrome of a bleeding tendency occurring only in males, and transmitted by apparently normal females (Otto, 1803).

The first successful treatment of haemophilia was achieved in 1840 by Lane, who demonstrated that transfusion of fresh, whole blood could be used to control a haemophilia associated bleeding episode (Lane, 1840). In 1911 Addis published a paper in which he showed that the prolonged clotting time of haemophiliac blood could be corrected by normal plasma *in vitro*, concluding that a plasma factor is absent from haemophiliac blood (Addis, 1911). This lead to the treatment of haemophiliac bleeding episodes with citrated plasma by Feisly in 1923 (Feisly, 1923). The nature of this plasma deficiency was later to be ascribed to antihaemophiliac globulin by both Brinkhouse and Quick in 1947 (Brinkhouse, 1947; Quick, 1947) and subsequently assigned the Roman numeral VIII (Wright, 1962).

The idea that haemophilia might be due to more than one type of defect first arose from the findings of Pavlovsky (1947). Pavlovsky reported that the blood from two haemophiliac patients was mutually corrective both *in vitro* and *in vivo*. This observation that there were multiple types of haemophilia was subsequently confirmed by Schulman & Smith (1952), Aggeler *et al* (1952) and Biggs *et al* (1952). These researchers independently described cases which exactly resembled

haemophilia, both clinically and genetically, but which were due to a deficiency not of factor VIII, but of a new previously unrecognised clotting factor. Aggeler *et al* called this new clotting factor 'plasma thromboplastin component' (PTC), and the disease 'PTC deficiency', while Biggs *et al* named the absent factor 'Christmas factor', and the disease 'Christmas disease' after their first patient. This absent factor was later assigned the Roman numeral IX.

Deficiency in factor VIII was subsequently called haemophilia A, and the absence of factor IX haemophilia B.

Interwoven with the history of haemophilia A and haemophilia B, is the autosomal clotting disorder von Willebrand's disease, which was first described in 1926, by von Willebrand. The disorder is caused by a deficiency of von Willebrand factor (vWF), a large glycoprotein found in platelets, plasma and endothelial cells. vWF is involved in the interaction of platelets with the vessel walls, therefore a defect or deficiency in the protein leads to a failure in primary haemostasis. Another function of vWF is to bind factor VIII, protecting it from proteolysis, and hence inactivation. Due to this interaction, a reduction in the level of circulating vWF leads to a reduction in factor VIII levels.

The therapy of haemophilia using whole blood or plasma transfusion was hampered by the minute concentrations of these coagulation factors, their short half life and hence the large volume of whole blood or plasma required. The normal plasma concentration of factor VIII has been estimated to be between 100 and 200 ng/ml (Fulcher & Zimmerman, 1982), and the concentration of factor IX approximately 5 μg/ml. Early attempts to isolate factor VIII from normal human plasma for the treatment of haemophilia A were only partially successful (Pohle & Taylor, 1937; Lozner *et al*; 1939: Lozner & Taylor, 1939). The year 1946 saw the first specific product for the treatment of haemophilia, factor VIII rich Cohn fraction 1 (Cohn *et al*, 1946). This classical method involved cold-ethanol precipitation of proteins from human plasma, yielding a product that contained approximately 5-fold purified factor VIII. Further precipitation of this cryoprecipitate by glycine (Wagner *et al*, 1964; Johnson *et al*, 1964) or polyethylene glycol (Johnson *et al*, 1966) and the application of these techniques to extraction from large plasma pools, saw the

advent of more potent, clinically useful factor VIII preparations. The material produced by these methods was remarkably stable and soluble, allowing it to be sterile filtered to 0.22 μm, frozen and lyophilised.

The first factor IX concentrates produced for the treatment of haemophilia B, were manufactured by calcium phosphate absorption of EDTA anticoagulated plasma (Pool & Shannon, 1965). However, EDTA plasma is unsuitable for the recovery of factor VIII. The introduction of citrate based anticoagulants in plasma collection, prompted the development of alternative procedures for the preparation of factor IX concentrates. Ion-exchange adsorption was used to purify factor IX from the supernatant which remains following cryoprecipitation. The anion exchanger diethylaminoethyl (DEAE) bound to Sephadex was used for this purpose. This method results in the several hundred fold purification of factor IX as well as other vitamin K dependent factors (factor II, factor X, protein C and protein S) (Casillias *et al*, 1969; Hoag *et al*, 1969; Middelton *et al*, 1973).

Even though some of these coagulation factor concentrates are enriched several hundred fold over plasma, less than one percent of the protein contained in these concentrates is actually coagulation factor. Consequently, haemophiliacs treated with these products are exposed to a large load of foreign proteins. In addition, the raw material for every lot of coagulation factor concentrate is a pool of plasma collected from up to 20,000 donors. This has exposed patients receiving these products to the risk of blood-borne viruses, such as the human immunodeficiency virus (HIV) and hepatitis B and C. These complications of therapy have made it necessary to develop highly purified, more efficacious and safer coagulation factor concentrates.

Over the past few years, advances in protein fractionation techniques such as ion-exchange chromatography, affinity chromatography and immunoaffinity chromatography have enabled manufacturers to produce highly purified plasma derived concentrates (Burnouf *et al*, 1991; Weinsein, 1989; Feldman *et al*, 1989; Hrinda *et al*, 1991). In addition, advances in recombinant DNA technology and the cloning of the factor VIII and factor IX genes (Gitschier *et al*, 1984. Toole *et al*, 1984; Choo *et al*, 1982; Kurachi & Davie, 1982) have facilitated the production of

recombinant coagulation factor concentrates (Boedeker, 1992; Gomperts *et al*, 1992). The production of highly purified and recombinant concentrates has revolutionised the treatment of haemophilia and vastly improved the efficacy and safety of these coagulation factor concentrates.

2. Haemophilia

2.1 Genetics of Haemophilia

Haemophilia is one of the most common inherited bleeding disorders in humans. Haemophilia A and haemophilia B are both X-linked recessive diseases, occurring almost exclusively in males. The frequency of haemophilia is approximately 1 in 5,000 male births (1 in 10,000 in the general population) (Rizza & Spooner, 1983). Factor VIII deficiency accounts for 85% of these cases, factor IX deficiency for a further 14%, the remaining cases being rare congenital disorders such as factor XI, X, VII and V deficiency. Because the defective factor VIII and factor IX genes are located on the X chromosome, haemophiliac males possess no normal gene and therefore manifest the clinical disorder. Females with haemophilia A or B are rare (Barrow & Graham, 1974). Cases include females homozygous for the defective gene (the offspring of a union of a haemophiliac male and a carrier female), and carrier females whose paternal X chromosome demonstrates functional abnormalities at the factor VIII locus, resulting in hemizygosity. These abnormalities of the X chromosome can occur by two mechanisims. The first is extreme lyonisation resulting in the preferential inactivation of the healthy paternal X chromosome (Nisen *et al*, 1986; Ingerslev *et al*, 1989; Nisen & Waber, 1989; Kling *et al*, 1991). The second mechanism is a genetic abnormality for haemophilia in a female with only one X chromosome, such as mosaicism for Turner's syndrome, genotype XX/XO (Gilchrist *et al*, 1965; Neuschatz & Necheles, 1973).

2.2 Laboratory Diagnosis of Haemophilia A and Haemophilia B

As previously mentioned, factor VIII and vWF are present in plasma as a complex, and factor VIII levels are dependent on vWF levels. Therefore, the laboratory investigation of patients suspected of having haemophilia A requires a distinction between vWF deficiency and factor VIII deficiency. Investigation of potential haemophilia A patients involves a factor VIII coagulant activity assay, a vWF

antigen assay and ristocetin cofactor activity assay. The vWF antigen assay measures the quantity of vWF in plasma. The ristocetin cofactor activity assay is a functional assay for vWF, which measures vWF supported agglutination of human platelets by ristocetin.

Patients with haemophilia A will have reduced factor VIII coagulant activity, but normal vWF levels and function, whereas patients with vWD (von Willebrand disease) will have reduced factor VIII coagulant activity, as well as reduced vWF levels or function.

Investigation of potential haemophilia B patients requires a factor IX coagulant activity assay. Haemophilia B patients will have reduced factor IX levels. However, other causes of reduced factor IX must be excluded, such as liver disease. Carriers of haemophilia A and B may have reduced levels of factor VIII and factor IX respectively, and may bleed excessively following trauma or surgery. In many carriers however the coagulation factor levels will fall within the normal range.

2.3 Clinical Severity of Haemophilia

The severity of the clinical features which manifest in haemophilia patients usually correlates well with the levels of factor VIII or factor IX activity in patient plasma. The condition in patients whose factor VIII or IX levels are less than 1% of normal (<0.02 U/ml) is classified as "severe". Patients whose factor VIII or IX levels are greater than 5% of normal but less than 40% (0.05-40 U/ml) are usually considered "mild" haemophiliacs. Patients whose factor VIII or IX levels fall between these two ranges are classified as "moderate" (Brettler & Levine, 1994; Roberts & Lozier, 1991). Approximately 50% of diagnosed cases suffer from severe haemophilia, moderate and mild cases accounting for around 25% each.

2.4 Clinical Features of Haemophilia

Both haemophilia A and B are characterised by repeated episodes of spontaneous bleeding or prolonged bleeding as a result of trauma. In severely affected patients spontaneous, haematomas and haemarthroses are typical. Mildly affected patients rarely suffer from haemarthroses, and only bleed excessively as a result of definite trauma. The clinical features demonstrated by moderately severe haemophiliacs lie somewhere between these two.

3. Factor VIII

3.1 Function of Factor VIII

The physiological response to blood vessel injury is the sequential activation of plasma proteases in the blood coagulation pathway. Activation of these plasma proteases leads to the localised generation of thrombin, and ultimately the conversion of soluble fibrinogen to insoluble fibrin and the formation of a fibrin clot. Historically the coagulation factor cascade has been considered to be composed of two parts, the extrinsic system which is initiated by the activation of factor VII by tissue factor, and the intrinsic system, which is initiated by the activation of factor XII by collagen, negatively charged surfaces and kallikrein. Tissue factor, expressed by cells at the site of haemostasis, is thought to be the most important initiator of the coagulation cascade.

Factor VIII is a critical participant in the blood coagulation pathway, Figure 1. Factor VIII is itself devoid of enzymatic activity, but acts as a cofactor to accelerate the activation of factor X by activated factor IX (factor IXa) (Fujikawa *et al*, 1974). Factor VIII is proteolytically activated by thrombin to yield activated factor VIII (factor VIIIa). It is this activated form of factor VIII, which in the presence of platelets and calcium, dramatically enhances the Vmax of factor X activation by factor IXa (Griffith *et al*, 1982; Neuenschwander & Jesty, 1988). This means that the reaction occurs slowly in the presence of platelets and calcium only and is greatly accelerated by factor VIIIa through a direct effect on the interaction of the enzyme factor IXa and the substrate, factor X.

3.2 Structure of Factor VIII

Factor VIII is one of the largest, least stable coagulation factors, and has a complex polypeptide composition. Understanding of the structure of factor VIII has arisen from two recent advances. The first was the further purification of factor VIII free of vWF, from factor VIII concentrates and human plasma by immunoaffinity

chromatography (Fulcher & Zimmerman, 1982; Fay *et al*, 1982; Rotblat *et al*, 1985; Ganz *et al*, 1988). Partial sequence data derived from this highly purified human factor VIII (Vehar *et al*, 1984) enabled the second advance to be made, the cloning of the human factor VIII gene, from which the primary structure of factor VIII was elucidated (Gitschier *et al*, 1984; Toole *et al*, 1984; Wood *et al*, 1984).

(i) Purified Factor VIII

Factor VIII purified from factor VIII concentrates consists of multiple polypeptides of approximately 80-210 kDa (Fulcher & Zimmerman, 1982; Rotblat *et al*, 1985). The largest molecular mass species found in plasma, being the single chain 330 kDa species (Rotblat *et al*, 1985). The purified form of factor VIII is a two-chain, metal-ion-stabilised complex which consists of a variable heavy chain of 90-210 kDa and a light chain of 80 kDa, Figure 2 (Fulcher *et al*, 1985; Andersson *et al*, 1986; Eaton *et al*, 1986; Fay *et al*, 1986). The metal ion in the factor VIII complex is presumed to be calcium (Fass *et al*, 1982; Andresson *et al*, 1986; Eaton *et al*, 1987). The nature and the site of the cation-interaction with factor VIII is unknown, but could involve either calcium induced conformational change favouring the binding of the light and heavy chains or direct metal ion-bridging of the subunits.

(ii) Recombinant Factor VIII

The deduced amino acid sequence of factor VIII, obtained from the cloned cDNA sequence, predicts a mature polypepetide of 2,332 amino acid residues with a calculated molecular weight 264,763 Daltons (Vehar *et al*, 1984). Sequence analysis also indicates 25 potential asparagine-linked glycosylation sites (Tuddenham *et al*, 1979). The addition of carbohydrate would account for the largest circulating form of approximately 330 kDa (Rotblat *et al*, 1985). The amino acid sequence also shows that factor VIII is a heterodimer of an 80 kDa light chain in association with a 90-210 kDa heavy chain, and that these chains are both derived from a larger precursor peptide.

Further analysis of the amino acid sequence revealed that the structure of factor VIII can be divided into three sets of domains; the triplicate A domains, a B domain and two C domains (Vehar *et al*, 1984). These occur in the order A1: A2: B: A3: C1: C2, Figure 2. The A domains show structural homology to ceruloplasmin, the copper binding plasma protein, suggesting that the A domains of factor VIII may be involved in metal ion binding (Church *et al*, 1984: Vehar *et al*, 1984). The two C domains which both occur in the C-terminus of the factor VIII light chain exhibit homology to the discoidin lectins (Vehar *et al*, 1984). Discoidins are capable of binding negatively charged phospholipids, implying that the light chain is perhaps involved in binding factor VIII to the cell surface. The B domain is encoded entirely by exon 14 of the factor VIII gene (Wood *et al*, 1984) and contains 18 of the 25 potential asparagine-linked glycosylation sites. The B domain displays no homology to other proteins. The amino acid sequence of factor V, another cofactor involved in the blood coagulation cascade, shows a high degree of homology to factor VIII. Indeed cofactors V and VIII exhibit 40% homology within the A and C domains, their B domains sharing no homology. The functional significance of the B domain is unknown. A recombinant factor VIII product has recently been developed in which the B domain is deleted and replaced by a short connecting sequence of the first 5 and the last 9 amino acids of the B domain. This factor VIII molecule has been reported to have full biological activity (Berntorp *et al*, 1995; Berntorp *et al*, 1995a; Fijnvandraat *et al*, 1995; Smeds *et al*, 1995). Clinical trials with the product are ongoing.

3.3 Interaction of Factor VIII with vWF

The interaction of factor VIII with the multimeric glycoprotein vWF is the best characterised of its interactions with other components of the haemostatic system. Following vascular injury, a series of events takes place which begins with platelet adhesion and aggregation, a process in which vWF participates by "gluing" platelets to exposed subendothelium (Weiss *et al*, 1978; Baumgartner *et al*, 1980). Another function of vWF is to act as a carrier protein for factor VIII stabilising it upon secretion (Kaufman *et al*, 1988). Based on these functions it is thought that vWF may bring factor VIII to the site of the vascular damage. vWF has also been reported to protect factor VIII from activation by factor Xa (Hamer *et al*, 1987) and inactivation by activated protein C (Koedam *et al*, 1988). vWF does not however protect factor VIII from thrombin proteolysis (Hamer *et al*, 1987).

Circulating levels of factor VIII are dependent upon complex formation with vWF. This is most evident in severe von Willlebrand's disease in which patients with complete deletion of the vWF gene have greatly reduced factor VIII levels (Ngo *et al*, 1988). Each vWF monomer of 250 kDa contains one factor VIII binding site (Lollar & Parker, 1987). Whether each binding site in a vWF multimer is capable of binding a factor VIII molecule is unclear, however, complexes in the molecular weight range of 1-10 x 10^3 kDa are formed. Monoclonal antibody binding studies have localised vWF binding to factor VIII to a region of highly acidic amino acids, residues 1673 to 1684 within the amino terminus of the factor VIII light chain (Foster *et al*, 1988).

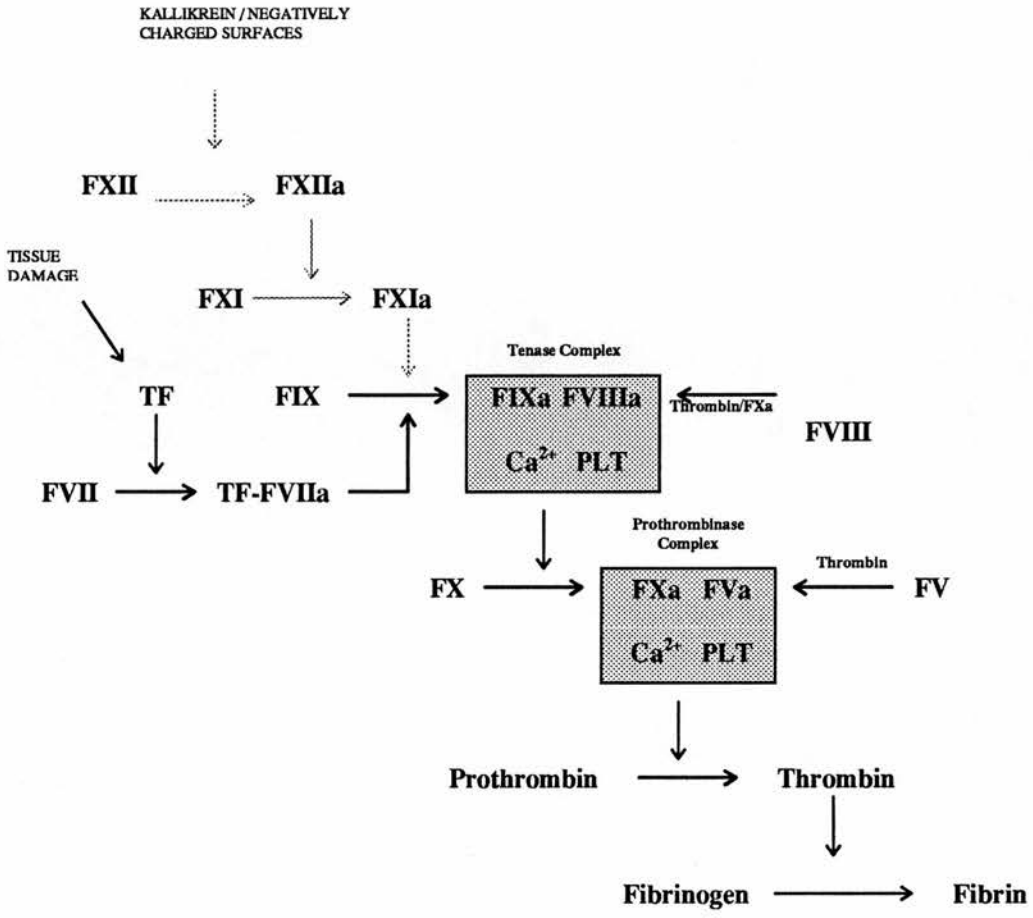


FIGURE 1: The Blood Coagulation Cascade.

Tissue factor, which is the initiator of the extrinsic pathway is thought to be the most important initiator of the blood coagulation cascade. The mechanism of initiation of the intrinsic pathway is represented by the light grey arrows. Activated coagulation factors are marked with a small case a.

Abbreviations: PLT = platelets; TF = tissue factor.

(Diagram adapted from Foster & Zimmerman, 1989)

3.4 Activation of Factor VIII

A number of blood coagulation serine proteases modulate the activity of factor VIII activity by specific proteolysis. The activation of factor VIII procoagulant activity by thrombin was first reported by Rapaport in 1963. Following this initial discovery, Hoyer & Trabold (1981) demonstrated that the activation of factor VIII was associated with a reduction in molecular weight as determined by gel filtration studies. Proteolysis of both the heavy and the light chain is necessary for activation, Figure 2. Thrombin cleavage of the heavy chain at arginine residue 740 generates a 90 kDa polypeptide which is subsequently cleaved at arginine residue 372 to generate two fragment of 50 and 43 kDa (Eaton *et al*, 1986). Thrombin also cleaves the 80 kDa light chain at arginine residue 1689 generating a 73 kDa polypeptide (Eaton *et al*, 1986). Each thrombin cleavage site is bordered by acidic amino acids, which may be thrombin binding sites. Mutation of arginine residues 372 and 1689 results in factor VIII molecules resistant to cleavage by thrombin and loss of cofactor function. Mutation at site 740 also yields a factor VIII molecule resistant to thrombin cleavage but has no effect on cofactor function (Pitman & Kaufman, 1988). This suggests that activation requires cleavage at sites 372 and 1689 but not at 740. It is thought that cleavage at site 1689 by thrombin is essential for the release of factor VIII from vWF, as this would result in the loss of the vWF binding site, residues 1673 to 1684. The importance of cleavage at residues 372 and 1689 for activation of factor VIII is demonstrated by haemophilia A patients who have mutations at either residue. In contrast to most haemophilia A patients, these patients have normal levels of factor VIII, as assessed immunologically, but no detectable factor VIII activity (Gitschier *et al*, 1988; O'Brien & Tuddenham, 1989).

Factor Xa also activates factor VIII in the presence of phospholipid (Davie *et al*, 1975). However, since only thrombin activates factor VIII in tissue factor activated plasma and because small amounts of thrombin are generated continuously *in vivo* (Bauer & Rosenberg, 1987) it is thought that thrombin is the sole activator of factor VIII *in vivo*.

3.5 Activated Factor VIII

Attempts to study activated factor VIII (factor VIIIa) have been hampered by its short half life. A study on thrombin activated porcine factor VIII suggested that the active form was a heterotrimer comprising 50 kDa, 43 kDa and 73 kDa fragments (Lollar & Parker, 1989; Lollar & Parker, 1991), this has been supported by Fay *et al* (1991). The present evidence indicates that activated factor VIII is a heterotrimer, which comprises heavy chain residues 1-372 (A1 domain) and 373-740 (A2 domain) plus light chain residues 1690-2332 (A3-C1-C2 domains) in a metal ion dependent complex, Figure 2.

3.6 Inactivation of Factor VIII

The molecular events which lead to factor VIII inactivation *in vivo* are unclear. Activated protein C (APC) has been shown to inactivate factor VIII by proteolysis. Activated protein C cleaves the 50 kDa factor VIII heavy chain at arginine residue 336 to generate a 45 kDa peptide, Figure 2 (Fulcher *et al*, 1984; Eaton *et al*, 1986; Walker *et al*, 1987). Mutagenesis of arginine residue 336 results in a factor VIII molecule with an increased specific activity. However, on thrombin activation, the 336 mutant did lose activity, suggesting that other cleavage sites are likely to be important for inactivation (Pitman & Kaufman, 1988)

Factor Xa has also been shown to inactivate factor VIII by proteolysis at position 1721-1722. More recently, it has been shown that incubation of factor VIII with physiological concentrations of highly purified activated factor IX resulted in the inactivation of factor VIII (O'Brien *et al*, 1992). This inactivation correlated with the cleavage of factor VIII at arginine 336 on the heavy chain, and at residues 1719-1720 on the light chain. The heavy chain cleavage at 336 was identical to that accomplished by APC, and the light chain cleavage similar to that by factor Xa. It is now believed that both APC and factor IXa inactivate factor VIII *in vivo*, this may explain why most individuals with protein C deficiency do not suffer from thrombosis (Miletich *et al*, 1987).

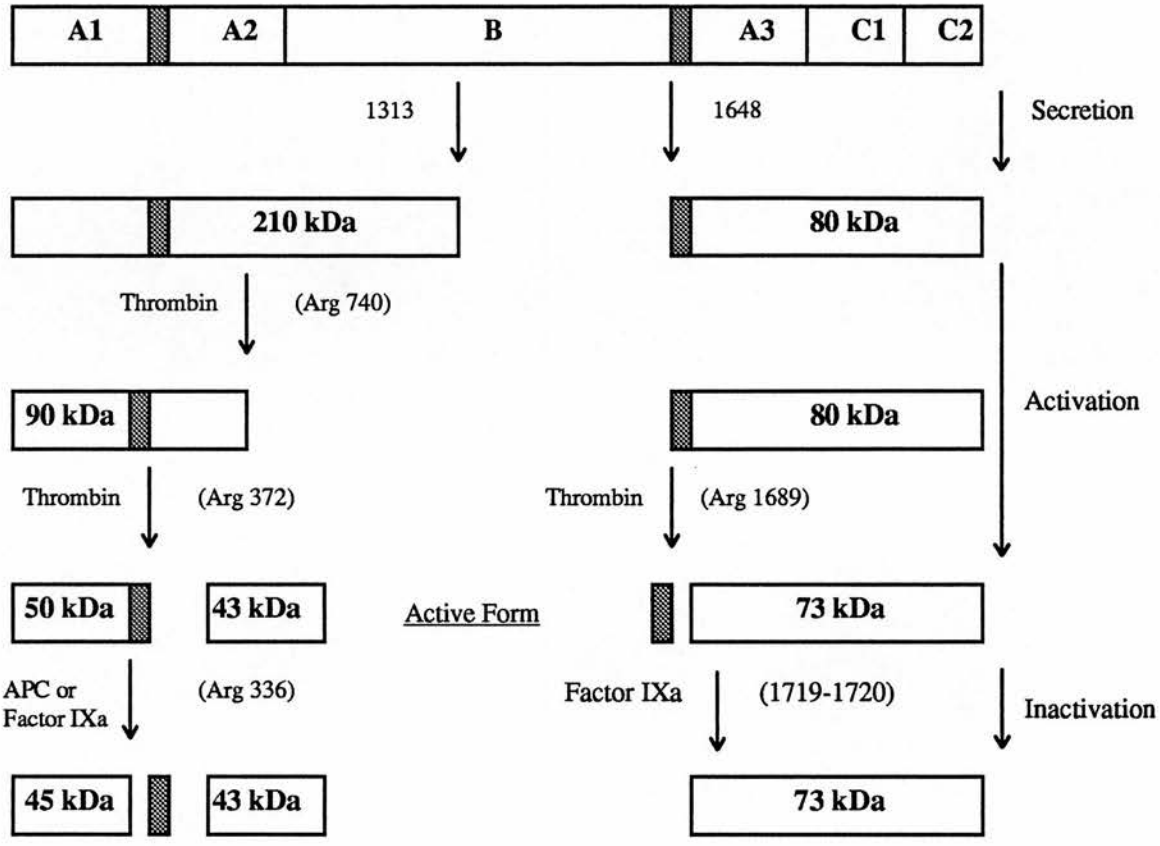


FIGURE 2: Domain Structure and Proteolytic Cleavage Sites of Factor VIII.

Factor VIII is cleaved by a protease(s) within the B domain to generate a 210 kDa heavy chain and an 80 kDa light chain, either during biosynthesis, secretion or during circulation. Activation of factor VIII coincides with proteolysis of the light and heavy chains by thrombin. The shaded areas correspond to regions of acidic amino acids. Abbreviations: Arg = arginine, APC = activated protein C.

Adapted from Pitman & Kaufman, 1988.

4. Factor VIII Gene Structure

The gene that codes for factor VIII is located at the distal end of the long arm of the X chromosome at band Xq28. The factor VIII gene is one of the largest genes known, comprising nearly 186 kbp, which represents approximately 0.1% of the X chromosome (Gitschier *et al*, 1984; Toole *et al*, 1984). The DNA encoding factor VIII is divided into 26 separate exons, the complete gene consisting of approximately 9 kb of exon and 177 kb of intron. The gene encodes a 9 kb messenger RNA, which translates into a precursor protein of 2332 amino acids residues plus a 19 amino acid signal sequence (Gitschier *et al*, 1984; Toole *et al*, 1984; Truett *et al*, 1986).

4.1 Factor VIII Gene Mutations

An international database of mutations in the factor VIII gene has been compiled, and is updated annually and published in Nucleic Acids Research. At the last update the haemophilia A database had 543 patient entries and 296 unique molecular events were recorded. These included 43 short deletions or insertions, 78 large deletions, 138 different amino acid substitutions and 24 nonsense mutations (Tuddenham *et al*, 1994). Approximately 25% of single base pair substitutions within the factor VIII gene involve cytosine to thymine transitions. This nucleotide substitution is now recognised to be the most frequent DNA change in the human genome, arising from spontaneous deamination of methylated cytosine residues. All of the above mutations recorded in the database account for only approximately 50% of the haemophilia A patient entries, no mutations being detected in the remaining 50% (Higuchi *et al*, 1991). The only defect observed in these patients was a lack of mRNA species that spanned intron 22 of the factor VIII gene (Naylor *et al*, 1992).

It is now known that this remaining 50% of haemophilia A cases result from major inversions at the tip of the long arm of the X chromosome. The inversions involve three highly homologous DNA sequences of approximately 9.5 kb. One copy is found within intron 22 of the factor VIII gene, the remaining two copies 500 kb proximal to the factor VIII gene. Intrachromosomal recombination can occur between the copy within intron 22 and either of the two proximal copies. This results in a separation and inversion of exons 1 to 22 of the factor VIII gene to a position 500 kb proximal of the remaining introns 23-26, and hence complete inactivation of the gene (Lakich *et al*, 1993; Naylor *et al*, 1993).

5. Factor IX

5.1 Function of Factor IX

The plasma protease factor IX plays a critical role in the blood coagulation pathway, Figure 1. The proenzyme factor IX is converted to its active form factor IXa, in the presence of calcium ions, by activated factor XI (intrinsic pathway) or by tissue factor complexed to activated factor VII (extrinsic pathway), Figure 1. Factor IXa's function is to activate factor X. Although information on this reaction is incomplete, factor IXa appears to bind to the activated cofactor VIIIa and, in the presence of calcium ions and platelets, to form a complex known as tenase. This tenase complex converts the proenzyme factor X to its enzyme form factor Xa. As previously discussed, factor VIIIa acts as a cofactor, increasing the Vmax of the activation of factor X by factor IXa (Griffith *et al*, 1982; Neuenschwander & Jesty, 1988).

5.2 Structure of Factor IX

Human factor IX circulates as a single polypeptide chain of 415 amino acids. It has a molecular weight of 55 kDa, of which approximately 20% is carbohydrate. Upon activation the single chain is cleaved forming a 45 kDa protein which consists of a heavy and a light chain, Figure 3. Two potential asparagine linked sites are found at residues 157 and 167, and an oligosaccharide residue is attached at serine 53.

The structure of factor IX can be divided into four distinct domains. The Gla domain extends from residues 1 to 46 and contains 12 γ-carboxyglutamic acid residues. A major function of this domain is calcium dependent phospholipid binding. In addition, residues 3 to 11 of this domain are also essential for binding factor IX to endothelial cells (Cheung *et al*, 1992).

Factor IX contains two epidermal growth factor (EGF) like domains, EGF-1 and EGF-2 (Stenflo, 1991). The EGF-1 domain comprises residues 47 to 84. The function of this domain is unclear. However, it does contain one high affinity

calcium binding site, which has been suggested to be involved in the conformational changes necessary for interaction with factor VIII and factor X. The EGF-2 domain comprises residues 85 to 124. The function of this domain is also unknown; however, it has been suggested that it is necessary for factor IXa incorporation into the tenase complex.

Residues 145 to 180 are known as the activation peptide, as this section is cleaved from the zymogen during factor IX activation. Although this section contains most of the carbohydrate, no specific function has been attributed to it.

The domain extending from residues 181 to 145, is known as the catalytic domain. This region contains an active site which is typical of all serine proteases, histidine 221, aspartate 269 and serine 365. Following cleavage of the activation peptide, the heavy chain amino terminal valine 181 forms an ion pair with asparagine 364. This ion pair formation positions the active site serine 365 for interaction with factor IX substrate, factor X (Hamaguchi *et al*, 1993).

5.3 Activation of Factor IX

Factor IX is the zymogen of a serine protease and has no enzyme activity in the single chain form. Factor IX is activated by the cleavage of two peptide bonds arginine 145 - alanine 146 (the α cleavage) and arginine 180 - valine 181 (the β cleavage). This results in the release of a short activation peptide, molecular weight 10 kDa, and the generation of activated factor IX, Figure 3. Although the activation peptide is cleaved from the zymogen during activation, it appears to remain non-covalently associated with the molecule. Activated factor IX has a molecular weight of 45 kDa and consists of a heavy and a light chain, connected by a disulphide bond.

In vitro, both factor XIa (Davie, 1987) or factor VIIa complexed to tissue factor (Osterund & Rapaport, 1977), in the presence of calcium are capable of cleaving bonds 145 to 146 and 180 to 181. It is not known how factor IX is activated *in*

vivo. Activation of factor IX has no requirement for a cofactor, unlike the activation of all other steps in the coagulation factor cascade involving the activation of a vitamin K-dependent protein (factor VII, factor X), Figure 1. Furthermore, the activation rates are relatively slow in comparison with other activation steps in the coagulation pathway. This inefficiency in factor IX activation is thought to be physiologically important in providing a control step in coagulation.

5.4 Inactivation of Factor IX

In vitro, factor IX is inactivated slowly by anti-thrombin III (Rosenberg *et al*, 1975). The potential role of plasma protease inhibitors other than anti-thrombin III in the clearing of factor IXa from the circulation is unknown.

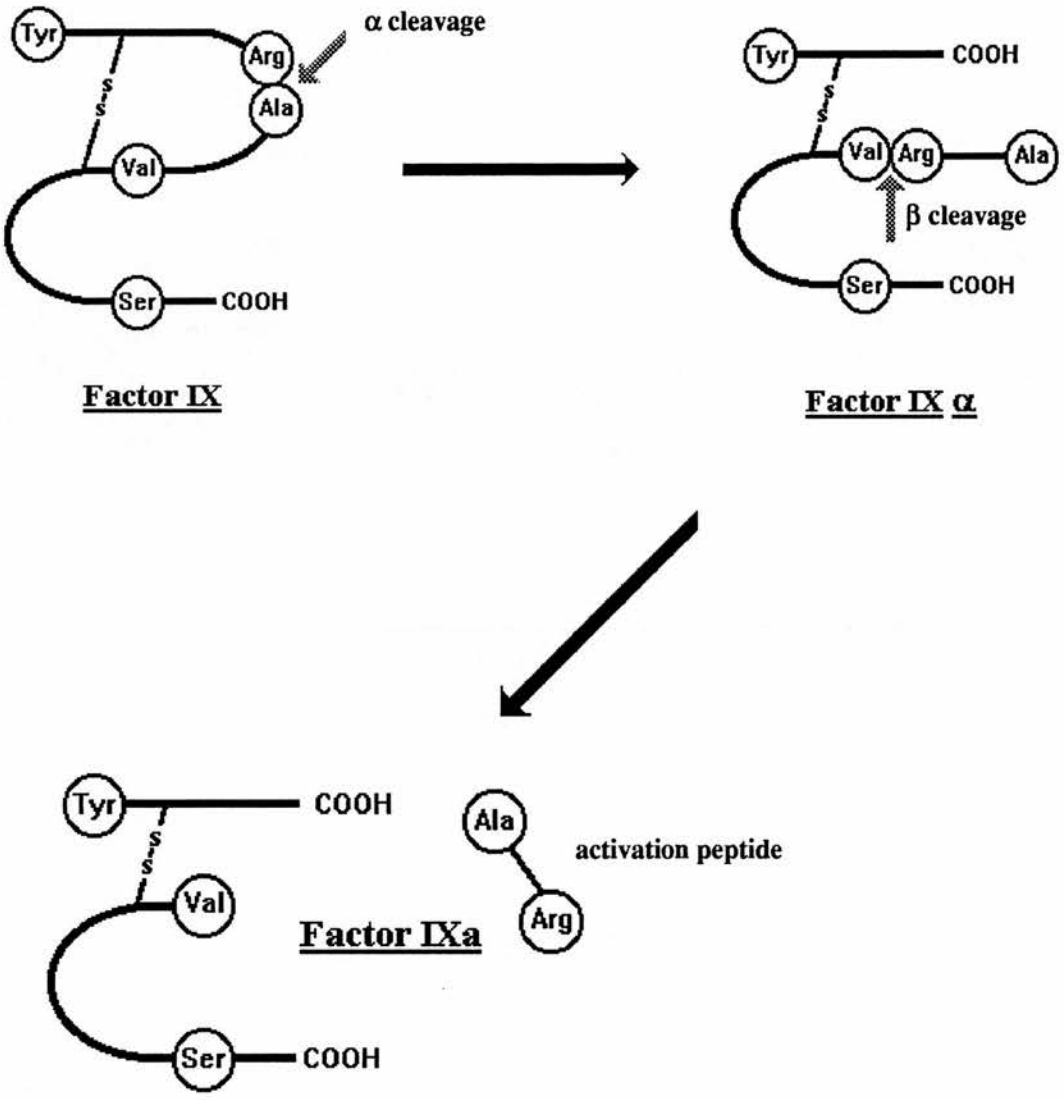


FIGURE 3: Activation of factor IX. Factor IXa or factor VIIa-tissue factor, cleaves the Arg^{145}-Ala^{146} bond to generate factor IXα, with the activation peptide remaining linked to the heavy chain. The Arg^{180}-Val^{181} bond is then cleaved to generate the active form factor IXa. Abbreviations: Ala = alanine, Arg = arginine, Ser = serine, Tyr = tyrosine, Val = valine.

(Adapted from High & Roberts, 1995)

6. Factor IX Gene Structure

The factor IX gene is located on the X-chromosome at band Xq27, about 40 megabases proximal to the factor VIII gene. The factor IX gene is approximately 33.5 kbp long, and comprises of 8 exons (Choo *et al*, 1982; Kurachi & Davie, 1982). The gene encodes a 1.4 kb messenger RNA, which translates into a precursor protein of 461 amino acid residues.

6.1 Factor IX Gene Mutations

As with factor VIII gene mutations resulting in haemophilia A, a database of factor IX gene mutations resulting in haemophilia B has been compiled. The last update of the haemophilia B database contained information on 1142 patients and reported the detection of 476 unique mutations. The mutations included 97 short deletions or insertions, 291 different amino acid substitutions and 106 mutations resulting in STOP codons. Within the factor IX gene approximately 32% of mutations resulting in termination codons or amino acid substitutions are a result of C to T transitions (Giannelli *et al*, 1994). This database does not include information reported in the literature on a number of patients who have complete or partial gene deletions or complex gene rearrangements.

7. Coagulation Factor Concentrates Available for the Treatment of Haemophilia

A range of products are available for the treatment of haemophilia, including various factor VIII and factor IX concentrates derived from human plasma and biosynthetic factor VIII products.

7.1 Feedstock Preparation

Human plasma for fractionation is collected from donors either as a whole blood donation, from which the cellular components are removed, or by plasmapheresis, where the cellular components are removed and returned to the patient. Plasma is collected in a solution of anticoagulant to prevent the donation from clotting. Following donation, the separated plasma is stored frozen for later processing. Since, virtually all factor VIII purification methods begin with the extraction of factor VIII from cryoprecipitate, freeze-thawing operations are of importance. Several stages of freeze-thaw process can be identified: freezing to -27°C and storage, the first stage of thawing of the plasma to -10°C and the second stage of thawing to a liquid state at 0-2°C. The plasma is then ready for entry into the fractionation process. Factor VIII is contained in the cryoprecipitate which forms when frozen plasma is thawed, and factor IX in the supernatant; therefore cryoprecipitate is used as a feed stock for factor VIII production and the supernatant for factor IX and other coagulation factor and blood product purification processes.

7.2 Precipitation

Precipitation was the protein separation technology initially used for fractionation of factor VIII, and ethanol selected as the precipitation reagent because of its volatile behaviour which enables it to be removed by drying technology (Edsall, 1947). Precipitation is a process whereby proteins are separated according to their solubility differences. The solubility of a protein is affected by a large number of parameters, some of these are specific to each protein and cannot be altered, e.g. molecular size, amino acid sequence and composition. The solubility of a protein is also altered by its enviroment. This may be changed easily, e.g. pH, temperature and ionic strength. Modifications of these variable parameters can be used to cause a selected protein or group of proteins to leave solution, hence precipitation.

Concentrates produced by this method are known as intermediate purity factor VIII concentrates as they contain less than 10 IU of factor VIII/mg of protein and contain many other contaminating proteins such as fibrinogen and fibronectin. This type of concentrate is being used less than formerly in the UK, as higher purity concentrates, produced by chromatography and recombinant DNA techniques are now readily available. Examples of this type of concentrate which are still licensed for infusion are Profilate OSD (Alpha Therapeutic Corporation) and Haemate P (Behringwerke).

7.3 Affinity Chromatography

Affinity chromatography is based on the adsorption principal. Adsorption is a technique whereby selected proteins or groups of proteins can be separated by selective binding from solution to a solid phase material. Solid phase reagents can be categorised according to the forces responsible for adsorption, for example charge interaction (ion-exchange), chemical interaction (affinity) and interaction with specific antibodies (immunoaffinity).

(i) Ion-exchange Chromatography

The principle of ion-exchange chromatography is that charged molecules adsorb to ion-exchangers reversibly, so that molecules can be bound and eluted by changing their ionic enviroment. Ion-exchangers can be used to separate molecules according to charge. Their chromatographic behaviour of proteins on an ion-exchange column is also sensitive to charge distribution and density and the molecular weight of the molecule. Separation on ion-exchangers is usually in two-stages; first the substance to be separated is bound to the exchanger using conditions that give stable and tight binding. The column is then eluted with buffers which compete with the bound material for the binding sites.

A method for the preparation of factor IX concentrates is ion-exchange using the anion exchanger DEAE. Factor IX concentrates produced by this method contain approximately 10-20 IU of factor IX/mg of protein. Examples of factor IX concentrates produced by this method are Defix (Scottish National Blood Transfusion Service, S.N.B.T.S.), and 9A (Bio Products Laboratory, B.P.L.). However, concentrates produced by this method also contain other vitamin K dependent procoagulant proteins such as factors II, VII and X. In addition trace amounts of other proteins such as factor XII, α_2-macroglobulin and ceruloplasmin have been detected in these concentrates (Pejaudier *et al*, 1987). Factor IX concentrates produced by this method are considered to be of intermediate purity as they contain many other proteins and have a low specific activity. Further purification following ion-exchange chromatography is necessary to remove these proteins. Immunoaffinity chromatography or a second anion exchange adsorption/desorption step, followed by affinity chromatography using dextran sulphate, has been used for this purpose. Factor IX preparations which are double purified by these methods are considered to be "high purity" products. An example of a concentrate produced by this method is AlphaNine SD (Alpha Therapeutic Corporation). Figure 4 outlines a typical plasma fractionation process used by S.N.B.T.S in the production of their high purity factor IX concentrate, High Purity IX.

The anion exchanger DEAE has also been used in the further purification of factor VIII from precipitate (Burnouf *et al*, 1991). By changing the pH or the ionic strength of the buffers used to elute the column, specific proteins can be purified; also, by careful selection of the solid phase matrix to which the DEAE is bound, further purification on the basis of molecular weight can be achieved. Factor VIII concentrates produced by ion-exchange have a specific activity of >100 IU of factor VIII/mg of protein and are considered to be high purity concentrates. Factor VIII concentrates of this type are now widely prescribed in place of those manufactured by conventional precipitation. Examples of this type of factor VIII concentrate are High Potency Factor VIII (HPVIII) now known as Liberate (S.N.B.T.S.) and Immunate (Immuno).

A typical plasma fractionation operation producing ion-exchange purified factor VIII is shown in Figure 4. The process outlined here is based on the plasma fractionation processes used by S.N.B.T.S. to produce the factor VIII concentrate HPVIII.

For the manufacture of factor VIII concentrate, the cryoprecipitate formed during the freeze-thawing processes is resuspended and the proteins separated by cold-ethanol precipitation. Cold ethanol precipitation is the dominant protein separation technique used in plasma fractionation (Kistler & Nitschmann, 1962; Holst *et al*, 1978). Several precipitation steps are necessary to ensure the removal of fibrinogen and fibronectin (Newman *et al*, 1971; Thorell & Blomback, 1984; Ng *et al*, 1986), which are poorly soluble adherent proteins which may hamper subsequent chromatographic and filtration operations. Following the precipitation of protein, residual proteins of the prothrombin complex are removed by adsorption to aluminum hydroxide (Newman *et al*, 1971). The separation of proteins by precipitation is complete when the solid and liquid phases have been separated. Centrifugation is used for this purpose. At this point, most manufacturers include a viral inactivation step such as solvent detergent treatment, pasteurisation or heat treatment. Subsequent to viral inactivation, chemicals used in solvent-detergent treatment or stabilisers used in pasteurisation must be removed by further purification. This may be achieved chromatographically, using either ion-exchange

or immunoaffinity adsorption or size exclusion chromatography. Since factor VIII is only a trace protein in plasma, highly purified preparations will contain only small amounts of protein. It is therefore necessary that the product be carefully formulated to minimise adsorption of factor VIII to the container surface and to make it compatible with the final stages of manufacture such as freeze drying and sterile filtration. This may be achieved by adding amino acids or human albumin to the product, increasing ionic strength, preventing adsorption to the container and providing a bulking agent for freeze drying (McIntosh & Foster, 1990).

(ii) Chemical Affinity Chromatography

Chemical affinity chromatography is based on the same adsorption principle as ion-exchange but, instead of utilising charge effects, affinity of the protein to certain chemicals is exploited.

Factor VIII can be further purified by passing precipitate through columns containing heparin coupled to cross-linked agarose. vWF has an affinity to heparin, and since vWF is bound to factor VIII, it is separated form other contaminating proteins. An example of a factor VIII product purified by this technique, which is licensed, is Alphanate (Alpha Therapeutic Corporation). Factor VIII produced by this method has a specific activity of 1000 IU of factor VIII/mg of protein. However it is unstable and requires the addition of human albumin as a stabiliser.

Factor IX purified by DEAE ion-exchange may also be further purified by chemical affinity to metal chelates bound to solid matrix columns, Bio Products Laboratory currently manufacture a concentrate by this method, 9MC.

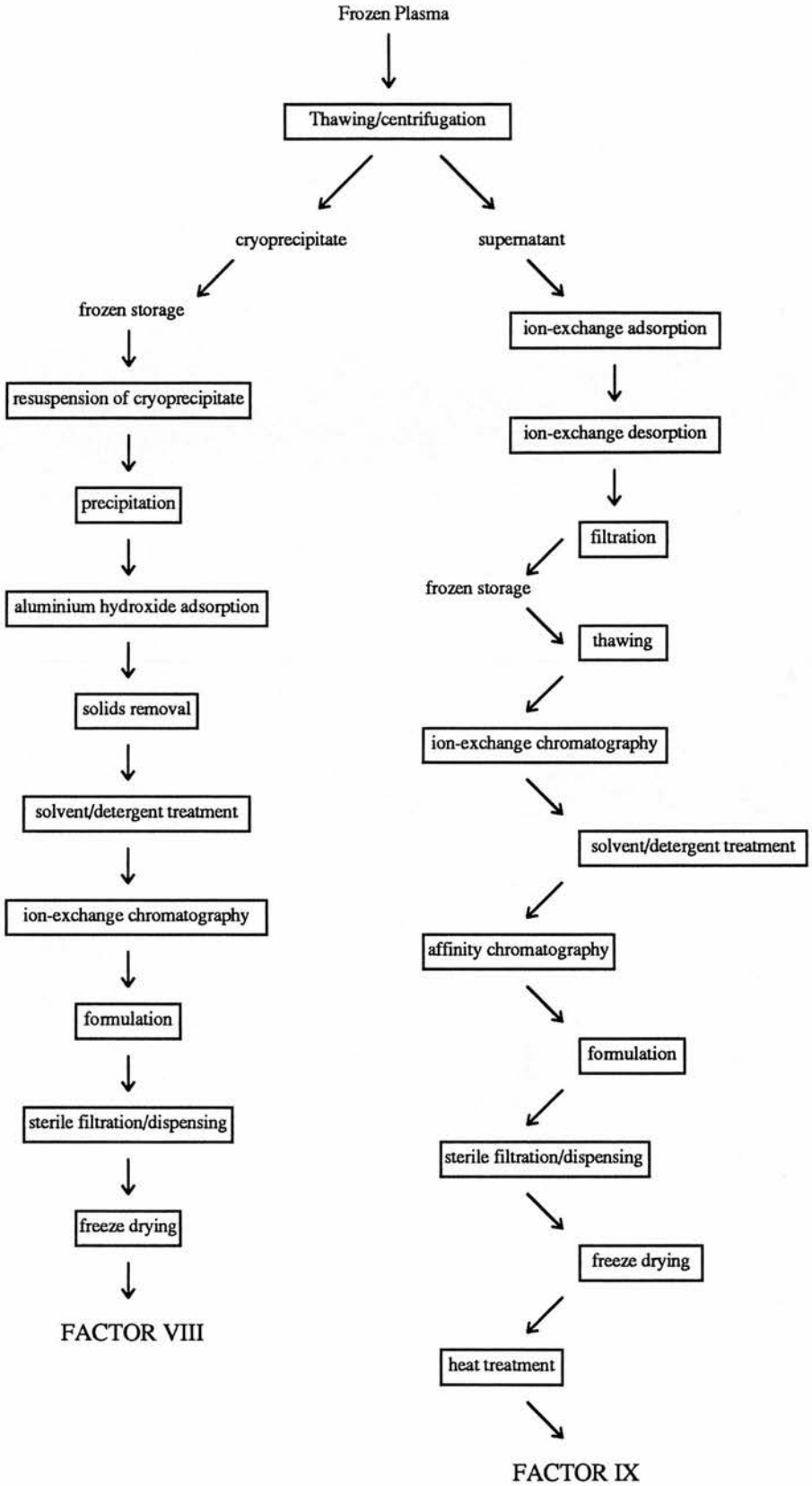


FIGURE 4: Typical plasma fractionation operation.

The fractionation procedures outlined here are based on processes at the Protein Fractionation Centre, Scottish National Blood Transfusion Service, Edinburgh.

(Adapted from Foster, 1994)

(iii) Immunoaffinity Chromatography

Factor VIII can be further purified from precipitate by using monoclonal antibodies directed against vWF (Monoclate P, Armour Pharmaceutical Company) or factor VIII (Hemofil M, Baxter Laboratories). When monoclonal antibody to vWF is used to purify factor VIII, the column is eluted with a calcium solution. Since calcium is a known stabiliser of factor VIII, it is gently dissociated from the vWF bound to the column. Following purification by the monoclonal antibody affinity column, the factor VIII containing elute is subjected to ion-exchange chromatography to remove any unwanted material, including murine antibody. Factor VIII purified by immunoaffinity is unstable, as it is purified without vWF, therefore human serum albumin is added as a stabiliser. Factor VIII concentrates purified by this method have a specific activity of approximately 3000 IU of factor VIII/mg of protein prior to the addition of albumin.

Factor IX can also be further purified from DEAE adsorption prepared concentrates by using a monoclonal antibody to factor IX. Only one concentrate is at present produced by this method, Mononine (Armour Pharmaceutical Company).

7.4 Recombinant DNA Techniques

Development of recombinant factor VIII began in 1984 when the factor VIII gene was characterised and cloned (Gitscher *et al*, 1984; Toole *et al*, 1984). Chinese hamster ovary (CHO) cells were chosen as the cells to produce recombinant factor VIII. The factor VIII cDNA was introduced into an expression vector and transfected into CHO cells. However, factor VIII biosynthesis by CHO cells is limited due to instability of the mRNA, inefficient secretion and the instability of the protein in the media. Attempts to improve efficiency of production have focused primarily on the development of cDNA constructs which lack most of the B domain. As previously mentioned, the B domain is not required for normal procoagulant function (Toole *et al*, 1986). Indeed, increased yields of factor VIII have been observed from CHO cells transfected with B domain-less cDNA. A recombinant

product without the B domain is at present undergoing clinical trials (r-VIII SQ, Pharmacia).

Factor VIII is purified from medium harvested from CHO cell cultures initially by immunoaffinity chromatography. The factor VIII eluted from the column is then further purified by ion-exchange which removes any residual contaminating components. Following this, the recombinant factor VIII has albumin added as a stabiliser. The specific activity of these products is more than 3000 IU of factor VIII/mg of protein before the addition of albumin.

Although the factor IX gene was cloned and sequenced in 1982 (Kurachi & Davie, 1982; Choo *et al*, 1982) a recombinant factor IX concentrate for clinical use has yet to be licensed. Many problems have hindered the production of recombinant factor IX. These problems relate to the many post-translational modifications which are required to produce active functional factor IX. Most expression systems are unable to perform the modifications, which include glycosylation, cleavage of the propeptide and carboxylation. Therefore, large amounts of non-functional protein are produced along with small amounts of functional protein. In addition, there are fewer patients with haemophilia B than haemophilia A and, therefore, not the same financial incentive to produce factor IX concentrates as there is to produce factor VIII concentrates.

7.5 Sterile Filtration and Freeze Drying

Concentrates prepared for infusion into patients must be sterile at the point of use. This is achieved by filtration of the finally formulated solutions through a series of filters, down to a final 0.2 μm membrane filter. Processing beyond this point, such as dispensing and freeze drying must all be carried out under sterile conditions. Preparations for use in patients must have a defined shelf life and must retain their biological activity for this length of time. Freeze drying of products fulfills these criteria. Freeze drying involves the separation of water from non-volatile constituents by sublimation from a frozen state (Nicholson, 1977). For this process the product is cooled to a temperature below which no significant liquid-solid

phase transition exists. The majority of the water is then sublimated from heating the frozen product under reduced pressure. When sublimation is complete further heat and pressure is applied to remove residual moisture. A final moisture content of approximately 2% is usually achieved.

As discussed above, there are many different types of coagulation factor concentrate available for the treatment of haemophilia. At present these concentrates are labelled as "intermediate purity", "high purity" and "very high purity" based on the specific activity, which is the amount of coagulation factor activity present per mg of protein (IU/mg). Specific activity of a product is determined at the point of manufacture when the coagulation factor is at its purest. However, there is much controversy surrounding this method of labelling.

Immunoaffinity purified products contain almost exclusively factor VIII and only trace amounts of fibrinogen and fibronectin. This type of product, following chromatography, has a specific activity of approximately 3000 IU/mg. However, these products are unstable as factor VIII is purified without its natural stabiliser vWF. In order to aid stablisation and administration, purified human serum albumin is added following elution from the immunoaffinity column. Factor IX products produced by immunoaffinity do not require the addition of albumin as a stabiliser.

As with immunoaffinity purified products coagulation factors produced by recombinant methods are unstable and therefore have albumin added as a stabiliser. These products, before the addition of stabiliser, have a specific activity of approximately 3000 IU/mg of protein. One particular recombinant factor VIII product which has no B domain is an exception, in that it is very stable, eliminating the need to add protein stabilisers.

Ion-exchange purified products contain factor VIII complexed to its stabiliser vWF and trace amounts of fibrinogen and fibronectin. On elution from chromatography columns, products are formulated directly and have a specific activity of >100 IU/mg. Other remaining proteins, including vWF in factor VIII concentrates, are

considered to be "contaminants" and the specific activity is reduced accordingly. The vWF content of ion-exchange purified factor VIII concentrates can vary greatly, from almost physiological concentrations to none at all (Fricke & Wong, 1989).

Controversy exists around the addition of stabilisers to some concentrates. As albumin is considered as a stabiliser, the specific activity quoted by some manufacturers is not altered after its addition. Following the addition of albumin the final specific activity is reduced from approximately 3000 IU/mg of protein to less than 10 IU/mg of protein. In addition, albumin used to stabilise these products is prepared from pooled human plasma and contains approximately 95% albumin, the remaining 5% being other proteins. The effect that these contaminating proteins should have on calculating specific activity remains under intensive debate. For the purpose of this study, coagulation factor concentrates are classified according to their method of manufacture:

- conventional fractionation/precipitation (intermediate purity)
- ion-exchange purified
- affinity purified
- immunoaffinity purified
- recombinant DNA technology manufactured

An exception to the above classification is factor IX concentrates prepared by DEAE ion-exchange but not further purified to remove other vitamin K dependent procoagulant proteins. These concentrates are considered to be of intermediate purity and are categorised accordingly. Factor IX concentrates further purified by ion-exchange are categorised so, as are those further purified by affinity.

8. Other Products Available for the Treatment of Haemophilia

As previously mentioned, patients with mild to moderate haemophilia A (factor VIII levels 2-40% of normal) only bleed excessively as a result of trauma or surgical procedures. Unlike patients with severe haemophilia who require coagulation factor replacement therapy, treatment with 1-Deamino-8-D-arganine vasopressin (DDAVP) is usually sufficient treatment for most bleeding episodes.

DDAVP is a synthetic analogue of vasopressin, which has been shown to prevent bleeding in patients with mild to moderate haemophilia A or von Willebrand Disease (Mannucci *et al*, 1977). Treatment with DDAVP is of no value to haemophilia B patients. The mechanism by which DDAVP increases factor VIII and vWF is unclear, but it is thought to stimulate the release of factor VIII from storage sites (Mannucci *et al*, 1981).

Treatment of mild to moderate haemophilia A by this method has reduced the need for blood products, thereby reducing the risk of transfusion acquired diseases such as, HIV and hepatitis B and C. DDAVP is administered to the patient either by intravenous or subcutaneous injection or intranasally, by the use of a simple atomiser.

9. Infectious Complications of Coagulation Factor Replacement Therapy

Coagulation factor concentrates have been associated with the transmission of viruses. Concentrates have been shown to have transmitted; hepatitis A virus (Mannucci, 1992), hepatitis B virus (Mannucci *et al*, 1988), hepatitis C virus (Watson *et al*, 1992), hepatitis D virus (Kingdon, 1970), B19 parvovirus (Azzi *et al*, 1992) and human immunodeficiency virus (HIV) (CDC, 1987a; CDC, 1987b). For this reason, measures to prevent viral transmission by coagulation factor concentrates have been introduced.

9.1 Virucidal Treatment of Coagulation Factor Concentrates

In order to minimise the risk of viral transmission by plasma derived coagulation factor concentrates, blood donors at low-risk of viral infection are selected and screening of blood donations has been implemented. At present in the UK all donations are screened for hepatitis B, hepatitis C and HIV-1 and -2. However, these methods alone are not sufficient to abolish viral transmission and a few virally infectious donations escape detection. Residual viruses that may have escaped detection at donor screening may be destroyed during concentrate manufacture by specific inactivation steps, such as pasteurisation, dry heat and solvent detergent treatment.

(i) Dry Heating

Terminal dry heating of lyophilised concentrates by manufacturers first began in the early 1980's. Concentrates were exposed to temperatures between 60°C and 68°C for up to 72 hours. Clinical evaluation of patients receiving these concentrates provided clear evidence that some concentrates "virally inactivated" at these temperatures still transmitted the hepatitis viruses (Colombo *et al*, 1985; Allain *et al*, 1986; Preston *et al*, 1985; Lush *et al*, 1988; Blacnchette *et al*, 1991, Pistello *et al*, 1991) and HIV (White *et al*, 1986; van den Berg *et al*, 1986; Mariani *et al*,

1987; Weisser, 1988; Dietrich *et al*, 1990; Remis *et al*, 1990; Williams *et al*, 1990). Dry heating at a higher temperature of 80°C for 72 hours is currently being used to virally inactivate the concentrates 8Y (factor VIII concentrate), 9A (factor IX concentrate) manufactured by BioProducts Laboratory (BPL) and Defix (factor IX concentrate) manufactured by S.N.B.T.S. Products heated in this manner show good coagulation factor recovery after the heating step (Foster & McIntosh, 1992). The advantage of heat treatment is that it can be carried out on the freeze dried product in its final container. Clinical studies on the viral safety of these concentrates have shown no transmission of the hepatitis viruses or HIV (Study Group of the UK Haemophiliac Centre Directors, 1988; Skidmore *et al*, 1990; Evans *et al*, 1991; Bennett *et al*, 1993). However, viral escape of the non-enveloped B19 Parvovirus has been reported (Williams *et al*, 1990a).

(ii) Pasteurisation

This virucidal method is currently used in the production of both intermediate purity concentrates, such as Haemate P (factor VIII concentrate) manufactured by Behringwerke) and concentrates purified by immunoaffinity, such as Monoclate P manufactured by Armour Pharmaceutical Company. This treatment involves the heating of the concentrate to 60°C in aqueous solution for a period of 10 hours. The loss of coagulation factor activity is one disadvantage of this method. There are no simple stabilising formulations for heating solutions of products which contain labile biological activities like factor VIII. Factor VIII activity can only be stabilised against pasteurisation by the addition of high concentrations of carbohydrate and glycine. However, such formulations cannot be used for final products and extra processing is required to remove the stabilisers after viral inactivation.

Studies on HIV transmission have shown that pasteurised concentrates have an excellent safety record; indeed a large retrospective study on 155 patients treated solely with pasteurised concentrate since 1979, reported that no HIV seroconversions had occurred in over 9 years (Schimpf *et al*, 1989). Furthermore,

when the immunoaffinity purified factor VIII concentrate Monoclate P was spiked with HIV, virus inactivation studies showed that pasteurisation reduced HIV by 10.5 $\log_{10}$ in comparison to an unheated control solution (Hrinda *et al*, 1990).

Patients treated with the pasteurised intermediate purity factor VIII concentrate Haemate P also showed no clinical or serological signs of infection with hepatitis, although some patients developed serological signs of infection with the heat resistant, non-enveloped B19 parvovirus (Azzi *et al*, 1992).

86 previously untransfused children who were treated solely with Haemate P were followed for up to 10 years. These patients were found to be consistently serologically negative for hepatitis C and showed no signs of infection with the virus (Kreuz *et al*, 1992).

Only nine cases independent of these studies have reported viral infection with hepatitis B or C (Brackmann *et al*, 1988; Schulman *et al*, 1992; Gerritzen *et al*, 1992; Shopnick *et al*, 1995). Therefore, even though the record of hepatitis safety of pasteurised concentrates reported by studies is excellent, there appears to be some risk of hepatitis infection even after pasteurisation.

(iii) Vapour Heating

This procedure was developed by Immuno and involves heating dried powder at 60°C for 10 hours in the presence of steam, under a pressure of 1190 mbar in a closed container.

Initial trials of 28 previously untransfused patients receiving concentrate virally inactivated by this method, reported that 4 out of the 28 patients had developed clinical or serological evidence for hepatitis B infection (Mannucci *et al*, 1988), 1 patient also developed hepatitis C (Mannucci *et al*, 1990). A subsequent study of 50 patients treated with vapour heated concentrate recorded no case of hepatitis or HIV infection (Mannucci *et al*, 1992a; Shapiro & the International Factor Safety Study Group, 1992). Whether these favourable results are due to an improvement in this viral inactivation method is unknown.

(iv) Solvent/Detergent

This method of viral inactivation involves the addition of an organic solvent tri (n-butyl) phosphate (TNBP) and a detergent (sodium cholate, Tween 80 or Triton X-100) at a bulk intermediate step in the manufacturing process. This method has been evaluated extensively in blood product applications and shows good product yield. Extensive processing steps after treatment ensure only trace amounts of these chemicals remain in the final concentrate. These chemicals lyse viruses with lipid envelopes, thereby inactivating large amounts of hepatitis B, hepatitis C, hepatitis D and HIV. This procedure has the advantages of being relatively simple and maintains high yields of biologically active factor VIII. Solvent/detergent treatment is now the most widely used of the viral inactivation procedures.

Several clinical studies have shown that no cases of hepatitis B, hepatitis C or HIV infection have occurred in a large number of previously untreated patients, treated exclusively with solvent/detergent inactivated product (Horowitz *et al*, 1988; Gazengel *et al*, 1988; Noel *et al*, 1989; Gonzaga & Boneker, 1990; Mariani *et al*, 1993; Addiego *et al*, 1992; Dipaolantonio *et al*, 1992). However, this viral inactivation procedure is ineffective on non-enveloped viruses, such as hepatitis A and B19 parvovirus. Infection with B19 parvovirus has occurred in a number of haemophiliacs infused with solvent/detergent treated concentrate (Azzi *et al*, 1992). In addition, B19 parvovirus DNA has been detected in solvent/detergent treated concentrates (Lefrere *et al*, 1994). An outbreak of infection with another non-enveloped virus, hepatitis A, has been recorded in 85 haemophiliacs from four countries, all treated with solvent/detergent factor VIII concentrate manufactured by Octapharma (Mannucci, 1992; Gerritzen *et al*, 1992a; Temperley *et al*, 1992; Peerlinck & Vermylen, 1993).

(v) Combined Virus Inactivation Techniques

In order to obtain absolute viral inactivation of both enveloped and non-enveloped viruses, manufacturers are beginning to use combined inactivation procedures. Chemical procedures such as solvent/detergent and physical methods such as heating or ultrafiltration are usually combined. Examples of products which undergo dual viral inactivation procedures are given below.

The factor IX concentrate Mononine manufactured by Armour, is virally inactivated using the chemical sodium thiocyanate and the inactive virus removed by ultrafiltration. Clinical trials assessing the viral safety of this concentrate are currently in progress. The ion-exchange purified concentrates from Immuno, Immunate STIM plus (factor VIII concentrate) and Immunine STIM plus (factor IX concentrate) are virally inactivated using two separate procedures, one chemical and one physical. The first is a Polyglycate treatment, which specifically inactivates retroviruses. These concentrates then receive terminal virus inactivation by vapour heating. Factor VIII concentrates which are both solvent/detergent treated then terminally heat treated are currently being developed by manufacturers including Alpha Therapeutic Corporation, Novo Nordisk, Octapharma and Aima.

(vi) Viral Safety of Recombinant Products

Coagulation factors produced by recombinant methods should carry no risk of transmitting blood borne viruses. However, there is a risk of infection from the human albumin used to stabilise these products and the bovine albumin used in the cell culture medium. There is also a theoretical risk that viruses associated with mammalian cell cultures may be pathogenic in humans. In order to minimise the risk of viral transmission from bovine or human albumin, manufacturers virally inactivate these preparations before use in the manufacturing process. Manufacturers claim that there is no risk of the hamster cells used in the manufacture of recombinant products carrying viruses harmful to humans. However, as an additional precaution, some manufacturers have introduced viral inactivation methods into the production process. Clinical studies on the viral safety of recombinant products are currently in progress.

10. Immunological Abnormalities in Haemophiliacs Receiving Coagulation Factor Replacement Therapy

The occurrence of HIV infection and AIDS in haemophiliacs (Ludlam, 1994), resulting from the use of contaminated coagulation factor concentrates, has led to an intensive study of the immune system of both HIV negative and HIV positive haemophiliacs. It is now recognised that clotting factor concentrates may cause immunological abnormalities, in haemophilia patients, distinct from those related to HIV infection. It has been proposed that these disturbances of the immune system are caused by the massive protein load (Ludlam *et al*, 1983), specific impurities (Wadhwa *et al*, 1994) or viruses (Evans *et al*, 1995; Pasi *et al*, 1995) in coagulation factor concentrates. These immune abnormalities which are observed *in vivo* and *in vitro* are summarised in Table 1 and Table 2 respectively.

10.1 Immune Abnormalities Observed *in vivo* in Haemophiliacs in the Absence of HIV Infection

As early as 1983 investigators demonstrated that haemophilia A patients have a reduced ratio of $CD4^+$ lymphocytes to $CD8^+$ lymphocytes (Ludlam *et al*, 1983; Menitove *et al*, 1983). This is partly due to a reduction in $CD4^+$ lymphocytes (Carr *et al*, 1984; Moffat *et al*, 1985; Antonaci *et al*, 1987; Cuthbert *et al*, 1992). However, this ratio is also lowered due to an absolute increase in the numbers of $CD8^+$ lymphocytes (Gamba *et al*, 1987; Freedman *et al*, 1987). Interestingly, haemophilia B patients treated with factor IX concentrates had T lymphocyte subsets comparable to that of normal controls (Carr *et al*, 1984; Cuthbert *et al*, 1992). No correlation was found between clotting factor consumption and T lymphocyte subset alterations (Carr *et al*, 1984).

$CD4^+$ lymphocytes of haemophiliacs receiving replacement therapy are affected not only quantitatively but also qualitatively. Cell mediated immunity, as assessed by the intradermal injection of recall antigens or dinitrochlorobenzene (DNCB), has revealed that HIV negative and HIV positive haemophiliacs are, to a large extent, unable to develop delayed hypersensitivity responses, which are an *in vivo* indicator

of $CD4^+$ T cell function (Brettler *et al*, 1986; Madhok *et al*, 1986; Cuthbert *et al*, 1992). Cell mediated immunity was found to be depressed to a greater extent in haemophilia A patients than in haemophilia B patients. A positive correlation between impairment of cell mediated immunity and annual factor VIII consumption has been demonstrated (Brettler *et al*, 1986; Madhok *et al*, 1986; Cuthbert *et al*, 1992). However, no such correlation was observed in the recipients of factor IX concentrates (Cuthbert *et al*, 1992).

It has been reported that haemophiliacs have an increased incidence of tuberculosis (Beddall *et al*, 1985) and recurrent hepatitis B (Williams *et al*, 1988). More recently, an HIV negative individual treated with factor VIII concentrate presented with oesophageal candidiasis, a condition suggestive of immune dysfunction (Watson *et al*, 1992a). This evidence suggests that the previously mentioned abnormalities of the immune system may be of clinical importance.

10.2 Abnormalities Observed *in vitro* in Cells of the Immune System Derived from Haemophiliacs Receiving Coagulation Factor Replacement Therapy

(i) T Lymphocytes

Peripheral blood mononuclear cells (PBMC) from haemophilia A patients show a reduction in response to activation with the T cell mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A), in comparison to control cultures (Froebel *et al*, 1983; Lederman *et al*, 1983; Moffat *et al*, 1985; Mahir *et al*, 1988).

T lymphocytes from haemophiliacs have also been shown to have a reduced capacity to produce interleukin 2 (IL-2) following PHA stimulation *in vitro* (Madhok *et al*, 1990; Madhok *et al*, 1991). This impaired production occurs irrespective of HIV antibody status, mean annual dose of factor VIII concentrate and liver disease severity.

Similarly, the production of interferon γ (IFN-γ) by T lymphocytes from HIV negative haemophiliacs is reduced (Ruffault *et al*, 1988).

More recently, Evans and colleagues (1995) studied the responses of lymphocytes derived from haemophiliacs to T cell mitogens. Three groups of haemophiliac boys and one control group were studied. Group 1 comprised 18 haemophiliac boys who were HIV negative and HCV negative. Group 2 comprised 12 HCV positive patients and Group 3 comprised 18 patients who were both HIV and HCV positive. Haemophiliacs negative for both HIV and HCV showed no significant reduction in lymphocyte proliferation in response to mitogens, when compared to the control group. Lymphocytes from boys of both groups 2 and 3 showed impaired response to mitogens compared to group 1 and the control group. No correlation between the factor VIII concentrate received and proliferative response was observed. The authors suggested that infection with viruses, such as HCV, may account for this impaired lymphocyte response and other similar lymphocyte function abnormalities.

(ii) B Lymphocytes

In normal individuals the pool of circulating B lymphocytes contains cells at different stages of activation; resting, partially activated, and fully activated cells which spontaneously secrete immunoglobulin. Studies on B lymphocytes from both HIV positive and HIV negative haemophiliacs have shown an increase in numbers of partially and fully activated B lymphocytes and consequently hypergammaglobulinaemia (Brevia *et al*, 1985). These findings were initially attributed to allogenic stimulation by foreign proteins which contaminate coagulation factor concentrates. Recently a study of B lymphocyte dysfunction in haemophiliacs demonstrated that the majority of HIV negative patients have *in vitro* immunoglobulin production similar to controls. Only in HIV negative patients with severe liver disease is increased spontaneous immunoglobulin production observed (Madhok *et al*, 1991). These results suggest that liver disease and not replacement therapy is the cause of B cell abnormalities in HIV negative haemophiliacs.

(iii) Monocytes

Monocytes from haemophiliacs receiving high amounts of coagulation factor concentrate have significantly lower expression of human leukocyte antigen-DR (HLA-DR), leukocyte function associated antigen-1 (LFA-1) and CR3 antigen (Roy *et al*, 1988). In addition, haemophiliac monocytes demonstrate reduced adhesion and an impairment in their response to chemotactic stimuli. No correlation between these observations and patient HIV status was found. However, a correlation between all of these changes and the annual dose of factor VIII concentrate was observed.

Furthermore, a deficiency in monocyte-T cell interaction after *in vitro* exposure to a bacterial antigen was observed in patients receiving factor VIII concentrates (Mannhalter *et al*, 1986). This functional defect in the early phase of the immune response, which has previously been described in patients with primary immunodeficiency (Eibl *et al*, 1982; Eibl *et al*, 1982a), may contribute to haemophiliacs' increased risk of opportunistic infections.

On the other hand, a recent study by Pasi *et al* (1995) suggested that impairment of monocyte function in haemophiliacs is due to viral infection rather than replacement therapy. Monocyte antigen presentation and cytokine production was studied in the same groups as Evans *et al*, 1995 (see previous section on T lymphocytes). The group which were both HIV and HCV negative showed no significant reduction in antigen presentation nor in IL-1 and IL-6 secretion, in comparison to the control group. Monocytes derived from the HCV positive, HIV negative, group demonstrated a significant reduction in antigen presentation, but no reduction in IL-1 or IL-6 secretion. Monocytes from HIV and HCV positive group showed both a reduction in antigen presentation and in cytokine secretion. The authors suggest that the reduction of IL-1 and IL-6 production is due to HIV infection and conclude that immune modulation in haemophiliacs is contributed to by chronic blood-borne virus infection.

(iv) Polymorphonuclear Leukocytes

Polymorphonuclear leukocytes form both HIV negative and HIV positive haemophiliacs have been shown to have impaired chemotactic, phagocytic and killing abilities. This is thought to be due to a significant decrease in the release of leukocyte inhibitory factor (LIF) and leukocyte derived chemotactic factor (LDCF) (Antonaci *et al*, 1987).

(v) Natural Killer Cells

Natural killer (NK) cells from haemophiliacs who received large infusions of intermediate purity clotting factor concentrate were assessed for reactivity to IFN-α, -β, -γ (Matheson *et al*, 1986). In repeated experiments it was demonstrated that haemophiliac NK cells had an impaired response to IFN-β and IFN-γ, but a normal response to IFN-α. Down-modulation of IFN receptors may cause this impaired response. It has been suggested that the down-modulation of these receptors is caused by chronic antigen stimulation as a result of the treatment of haemophilia with intermediate purity coagulation factor concentrates, which contain many foreign proteins. There was no correlation of these findings with HIV status (Matheson *et al*, 1986).

10.3 Effects of Coagulation Factor Concentrates on *in vitro* Immunological Assays

(i) Lymphocyte Proliferation

In vitro studies have demonstrated that intermediate purity factor VIII and factor IX concentrates can inhibit the proliferation of lymphocytes in a dose dependent manner, in response to the mitogens PHA and Con A. Reductions in PHA- and Con A-induced transformation occur when certain concentrates are incubated with normal human PBMC preparations (Froebel *et al*, 1983; McDonald *et al*, 1985; Lederman *et al*, 1986). Limited data are available regarding the effects of products

prepared by chromatography, immunoaffinity or recombinant techniques. However, what information there is suggests as purity increases the inhibitory effect decreases (Schreiber *et al*, 1987; Hay & McEvoy, 1989; Vermot-Desroches *et al*, 1992).

Intermediate purity concentrates have also been shown to impair lymphocyte proliferation in response to recall antigens (McDonald *et al*, 1985; Lederman *et al*, 1986) and to the monoclonal antibody OKT_3 (Wang *et al*, 1985).

Inhibition of lymphocyte proliferation by coagulation factor concentrates in the absence of mitogens, recall antigens or OKT_3 has not been observed.

Mixed lymphocyte reactions have been performed in the presence of coagulation factor concentrates. These assays involve stimulating PBMC with allogeneic lymphocytes in the presence of coagulation factor concentrates. The results from different research groups employing this assay system are conflicting. Batchelor *et al* (1992) reported that intermediate purity factor VIII and factor IX concentrates enhanced the proliferation of lymphocytes in response to allogenic cells. Enhanced proliferation was not observed with monoclonal antibody purified preparations. However, an ion-exchange purified concentrate enhanced proliferation in this assay system, though to a lesser extent. Those preparations which enhanced proliferation did not do so in the absence of the allogenic stimulator cells, suggesting the presence of co-mitogenic factor(s) in some concentrates. Enhancement of proliferation is, paradoxically, compatible with inhibition of specific immune function, including delayed-type skin reactions (Ascher & Sheppard, 1990). If these coagulation factor preparations were to enhance lymphocyte activation *in vivo* in response to naturally occurring immune stimuli or those present in concentrates, there may be a decreased response to other antigens.

In contrast, three other groups reported that factor VIII concentrates inhibited allogenic mixed lymphocyte reactions (Lederman *et al*, 1986; Schreiber *et al*, 1987; Hay & McEvoy, 1989). However, the results from these groups cannot be compared to those of Batchelor *et al*, as the concentrates studied were different and no technical details of culture conditions used were provided by Lederman *et al* (1986), Schreiber *et al* (1987) or Hay & McEvoy (1989).

(ii) Interleukin 2 Production

IL-2 is a potent T-cell growth factor, whose secretion is thought to be important in the development of many immunological functions. *In vitro* studies have shown that coagulation factor concentrates can inhibit IL-2 secretion by mitogen stimulated human lymphocytes or Jurkat tumor cells (human lymphoblastoid T cell line) (Lederman *et al*, 1986; Thorpe *et al*, 1989; Madhok *et* al, 1990; Wadhwa *et al*, 1992; Wadhwa *et al*, 1994). Thorpe *et al* investigated the effects of a range of factor VIII and factor IX concentrates on IL-2 secretion. The results demonstrated that intermediate purity factor VIII concentrates inhibited IL-2 secretion, whereas monoclonal antibody purified factor VIII concentrates did not. Factor IX concentrates purified by DEAE cellulose chromatography also inhibited IL-2 secretion. These findings were supported and expanded by Wadhwa *et al* (1992 & 1994) whose results demonstrated that some, but not all, intermediate purity factor VIII concentrates and those purified by ion-exchange chromatography displayed similar inhibitory characteristics, whereas monoclonal antibody purified and recombinant factor VIII concentrates did not cause any inhibition of IL-2 secretion.

Attempts have been made to correct the inhibitory effect of some factor VIII concentrates on lymphocyte mitogen responses by adding exogenous IL-2 (Hay *et al*, 1990). Although the addition of 250 IU/ml of IL-2 was observed to increase lymphocyte mitogenic responses at all concentrations of factor VIII, it did not completely reverse the inhibitory effect. This suggests the inhibition of mitogen-stimulated lymphocyte transformation is not due to the suppression of IL-2 secretion alone.

This is supported by Wadhwa *et al* (1992) who observed that the addition of large amounts of recombinant IL-2 to PHA-stimulated lymphocytes could not entirely reverse the inhibition of IL-2 receptor expression by factor VIII concentrates. This led them to conclude that the inhibition of lymphocyte inhibitory mitogenic responses by factor VIII concentrates is due to more than one mechanism.

T lymphocytes produce IL-2 in response to antigen stimulation and IL-1 secreted by antigen presenting cells (Smith, 1988). Since, IL-2 production by Jurkat cells proceeds independently of IL-1, it is unlikely that the inhibition caused by factor VIII and factor IX concentrates is mediated through interference with the response to, or production of, IL-1 (Lederman *et al*, 1986).

(iii) Activation Marker Expression

Incubation of PHA-stimulated lymphocytes with intermediate purity factor VIII concentrates causes a reduction in the expression of some cell surface activation markers. The expression of early activation markers CD25 (IL-2 receptor) and CD71 (transferrin receptor) and the late activation marker HLA-DR, on both $CD4^+$ and $CD8^+$ lymphocytes, is reduced on incubation with intermediate purity concentrates (Hay *et al*, 1990; Wadhwa *et al*, 1992). CD25 expression is the most profoundly affected, a 95% reduction being observed in the presence of a particular factor VIII concentrate (Hay *et al*, 1990). Less marked changes were observed in CD71 and HLA-DR expression.

The effects of an ion-exchange purified factor VIII concentrate on the expression of activation markers was also studied by Vermot-Desroches *et al* (1992). After exposure of PBMC to the ion-exchange product, a reduction in the induction of CD25, CD71 and HLA-DR expression was observed. In addition, there was reduced up-regulation of the late antigens CD38 (marker for activated T cells) and CD11a/CD18 (LFA-1α and LFA-1β respectively). Incubation of PBMC with this factor VIII concentrate did not influence the expression of the very early antigen CD54 (inter-cellular adhesion molecule; ICAM-1). The authors suggest that this product inhibits lymphocyte proliferation by interacting at a precise early step in the T cell activation process.

It has been proposed that inhibition of IL-2 secretion is a result of the down regulation of CD25 (IL-2 receptor) by component(s) in some factor VIII concentrates, as addition of exogenous IL-2 failed to correct the inhibitory effects of the concentrates on lymphocyte function (Hay *et al*, 1990).

On the other hand, Wadhwa *et al* (1992) suggested that it is the suppression of IL-2 secretion that causes the down regulation of CD25 expression. Since addition of exogenous IL-2 does partially reverse concentrate inhibition of PHA-induced up regulation of CD25. The authors suggest that factor VIII concentrates which inhibit CD25 expression do so by partially inhibiting IL-2 secretion, which results in impaired up-regulation of CD25.

(iv) Monocyte Function

The monocyte system is critical for the elimination of microbes (David, 1987). The incubation of monocytes from healthy individuals with certain factor VIII concentrates has been shown to affect the phagocytic function of monocytes, by reducing the expression of IgG Fc receptors in the membrane (Eibl *et al*, 1987; Mannhalter *et al*, 1988). This down-modulation results in an impairment of bacterial killing and a decrease in the generation of free oxygen radicals. The reduction in expression of IgG Fc receptors is comparable to that achieved after pre-treatment of monocytes with heat-aggregated IgG, a known down-modulator of Fc receptors (Mannhalter *et al*, 1987). This immune modulating activity is thought to be attributable to a very high molecular weight compound (molecular weight >1,270 kDa), containing IgG, IgM and traces of factor VIII.

Since the transmission of viral infections by clotting factor concentrates has complicated the treatment of haemophilia, heat treated concentrates are widely used. Concern was expressed that this type of virucidal treatment might cause the formation of aggregates and increase adverse effects (Bird *et al*, 1985). However, the degree of impaired monocyte function is unrelated to the mode of virus inactivation (Mannhalter *et al*, 1988; Pasi & Hill, 1990). Pasi & Hill (1990) nevertheless demonstrated that product purity does influence the degree of monocyte Fc dependent phagocyte function and that, as product purity increases, the observed inhibitory action of the concentrate fell.

The impairment of monocyte phagocytic function by some factor VIII concentrates may lead to a defect in antigen handling (Mannhalter *et al*, 1986). This, together with the other *in vitro* observations, may account for the previously mentioned susceptibility of haemophiliacs to viral infection.

(v) Natural Killer Cells

The effect of an intermediate purity factor VIII concentrate on natural killer cell activity was investigated by Lederman *et al* (1986). Natural killer activity against tumour antigens was assessed by a standard chromium release assay. The concentrate was found to have minimal effect on natural killer activity, even at high concentrations.

11. Immunomodulatory Components in Coagulation Factor Concentrates Affecting Lymphocyte Mitogenic Responses

11.1 Dialysable, Low Molecular Weight Inhibitory Component

It has been suggested that a dialysable, low molecular weight component of coagulation factor concentrates is either partially or fully responsible for the observed inhibition of lymphocyte proliferation *in vitro*.

McDonald *et al* (1985) reported that inhibition of proliferation of PBMC by intermediate purity coagulation factors was entirely due to a dialysable component. Proliferation of lectin or recall antigen stimulated PBMC was inhibited by a factor VIII concentrate and a factor IX concentrate. Dialysis of these concentrates led to a complete loss of inhibitory activity. In addition, when the buffers used in the preparation of these coagulation factors were tested for their ability to inhibit PBMC proliferation, the degree of inhibition was similar to that obtained with the concentrates. A common component of these buffers was citrate. Indeed sodium citrate alone was an effective inhibitor in their assay systems. A subsequent report by Wang *et al* (1986) supported the above findings by demonstrating that the inhibitory effect an intermediate purity factor VIII concentrate had on lymphocyte proliferation was completely abrogated by dialysis.

Another group investigating the effects of an ion-exchange purified factor VIII concentrate on PHA-stimulated PBMC proliferation demonstrated that a significant portion of the inhibitory activity was due to the presence of a dialysable, low molecular weight component (Vermot-Desroches *et al*, 1992). Vermot-Desroches *et al* also reported that the inhibitory influence that this product had on markers of lymphocyte activation was modified following dialysis. The expression of early activation markers CD25 and CD71, and the late activation markers CD11a/CD18, CD38 and HLA-DR was inhibited by this factor VIII concentrate. After dialysis, no significant inhibition of PHA-induced expression of these markers was observed.

The effects that dialysis has on concentrates which affect the *in vitro* secretion of IL-2 by lymphocytes was investigated by Wadhwa et al (1992). A range of factor VIII products of varying purity and process of manufacture were dialysed and their

effects on IL-2 secretion studied. The results demonstrated that only 5-20% of the inhibitory activity of intermediate purity products could be removed by dialysis. In contrast, 40-60% of the inhibitory activity of ion-exchange purified products was removed by dialysis. The authors suggest the greater dialysable inhibitory component in ion-exchange products is due to stabilisers such as citrate being added to these products to prevent degradation during viral inactivation.

11.2 Fractionation of Immunomodulatory Factor VIII Concentrates

Gel filtration of a conventional intermediate purity factor VIII concentrate which inhibited PHA-stimulated lymphocyte proliferation, revealed two peaks of inhibitory activity (Lederman *et al*, 1986). One of molecular weight 200 kDa, which co-migrated with factor VIII coagulant activity and antigen, another of molecular weight 60 kDa found in fractions devoid of factor VIII coagulant activity or antigen. The authors also reported that fractions of intermediate molecular weight enhanced lymphocyte proliferation. These fractions did not enhance lymphocyte proliferation in the absence of PHA. The authors suggest that the 200 kDa species is either factor VIII itself or that a substance bound or co-migrating with factor VIII inhibits lymphocyte proliferation. The smaller protein was also unidentified.

Results of size exclusion chromatography on factor VIII concentrates which inhibited IL-2 secretion by lymphocytes revealed similar results (Wadhwa *et al*, 1992). The results showed that most of the inhibitory activity was located in a protein fraction with a molecular weight of approximately 200 kDa. A much smaller peak of inhibitory activity was also observed at a molecular weight of approximately 60 kDa. In some concentrates, a strongly inhibitory component eluted at the total column volume, the expected elution point for buffer salts.

The 200 kDa fraction contained large amounts of fibrinogen and fibronectin. However, the authors suggest that these are unlikely to be responsible for the inhibitory action, since the purified proteins when tested had no effect on IL-2 secretion. Fractions from non-inhibitory factor VIII concentrates showed no inhibitory effects.

Thorpe *et al* (1989) analysed the protein composition of factor VIII concentrates of varying purity and manufacture by gel permeation chromatography, using fast protein liquid chromatography (FPLC). Despite differences in inhibition of IL-2 secretion by the various concentrates, the size exclusion chromatography profile was similar in both inhibitory and non-inhibitory concentrates. Comparison with purified proteins showed a fibrinogen peak and an albumin peak. Factor VIII itself was recovered in the void volume. A peak with a molecular weight <10 kDa was recovered and assumed to be low molecular weight stabilisers.

11.3 Transforming Growth Factor Beta

It has recently been demonstrated that transforming growth factor beta (TGF-β) is major contaminant of some factor VIII concentrates (Wadhwa *et al*, 1994). Levels of TGF-β detected in factor VIII concentrates by bioassay were found to correlate strongly with their ability to inhibit IL-2 secretion by a PHA-stimulated T cell line. Furthermore, the authors stated that the addition of a specific TGF-β antibody reversed the inhibitory effect of some concentrates on IL-2 secretion.

The authors suggest that some of the *in vitro* effects that coagulation factor concentrates have on cells of the immune system, resemble the actions of TGF-β. Indeed, the proliferation and IL-2 secretion of mitogen stimulated lymphocytes is inhibited by both purified TGF-β (Fox *et al*, 1992; Ahuja *et al*, 1993) and some coagulation factor concentrates (Froebel *et al*, 1983; McDonald *et al*, 1985; Lederman *et al*, 1986; Vermot-Desroches *et al*, 1992; Thorpe *et al*, 1989; Madhok *et al*, 1990; Wadhwa *et al*, 1992). In addition, TGF-β has been demonstrated to impair monocyte function (Tsunawaki *et al*, 1988), as have some factor VIII concentrates (Eibl *et al*, 1987; Mannhalter *et al*, 1987; Pasi & Hill, 1990).

TGF-β comprises a family of multifunctional polypeptides that regulate the cellular growth and differentiation of a wide variety of cells (Sporn *et al*, 1987; Massagué, 1990). The TGF-β family in man consists of TGF-β1, TGF-β2 and TGF-β3. TGF-β is released from cells, and circulates in plasma as a latent complex which does not

bind to receptors (Pricher *et al*, 1986; Wakefield *et al*, 1988; Massagué, 1990). Several configurations of latent TGF-β have been identified, including the large latent complex and the TGF-β-α_2-macroglobulin (α_2M) form (O'Connor-McCourt & Wakefield, 1987; Wakefield *et al*, 1988; Huang *et al*, 1988).

Wadhwa *et al*, suggest that the presence of TGF-β in factor VIII concentrates, in both active and latent forms is unsurprising, since all the concentrates studied were plasma derived. They suggest that feedstock plasma may contain residual platelets, which are the predominant producers of TGF-β, and that the handling and storage of plasma prior to cryoprecipitation could cause the lysis of platelets and the release of latent TGF-β; subsequent purification procedures and viral inactivation steps yielding the active cytokine.

In summary, from the evidence in the literature it would appear that the main candidates responsible for the *in vitro* immune modulation by some coagulation concentrates, are TGF-β and a dialysable low molecular weight component. Whether the presence of these contaminants in coagulation factor concentrates accounts for all the immune modulating activity observed *in vitro* at present remains undetermined.

12. Comparison Studies

The majority of the evidence in the literature suggests that the greater the purity of the product, the less immune modulation it is likely to cause *in vitro*. It has been postulated that high purity factor VIII concentrates, which contain less allogenic proteins than intermediate purity concentrates, might induce less deterioration of the immune system of HIV positive haemophiliacs and reduce the immune modulation in HIV negative haemophilacs.

(i) Studies in HIV Positive Haemophiliacs

In order to evaluate the above hypothesis, Brettler *et al* (1989) studied the decline in $CD4^+$ counts in HIV positive haemophiliacs receiving concentrates of varying purity. $CD4^+$ cell decline have previously been demonstrated to have a predictive value for the onset of AIDS (Eyster *et al*, 1987; Philips *et al*, 1991). Seven HIV positive haemophiliacs receiving a monoclonally purified product were followed for a period of 2 years. These patients' $CD4^+$ counts remained stable over the period of study. Patients receiving intermediate purity factor VIII were chosen as retrospective controls. The $CD4^+$ counts of the control group also remained stable. The response to skin test antigens was the only difference observed between the two groups. The group treated with monoclonally purified concentrate showed a decrease in skin anergy. In contrast, the group treated with intermediate purity concentrate showed an increase in skin anergy. The authors suggested immune function in HIV positive patients may be stabilised by more highly purified concentrates.

This study prompted a number of similar investigations into the $CD4^+$ counts and cellular immunity of both HIV positive and HIV negative haemophiliacs receiving replacement therapy. Several reports support the hypothesis put forward by Brettler *et al* that monoclonally purified concentrates stabilise the immune system of HIV positive haemophiliacs.

de Biasi *et al* (1991) studied 20 HIV positive haemophiliacs. Half were treated with intermediate purity Kryobulin TIM3, the remainder treated with immunoaffinity

purified Hemofil M. Following a two year study period, the authors reported a significant steady decline in $CD4^+$ counts in the group treated with intermediate purity concentrate. The $CD4^+$ counts of the group receiving monoclonally purified concentrate showed no significant change. Furthermore, in the group being treated with monoclonally purified concentrate, four out of six patients, who on entry to the study were anergic acquired reactivity to skin testing. Other similar studies, comparing immuoaffinity purified concentrates to intermediate purity concentrates, supported the findings of de Biasi *et al* (Seremitis *et al*, 1990; Goldsmith *et al*, 1991; Seremitis *et al*, 1993; Goedert *et al*, 1994). Two of these studies however demonstrated that, despite a stabilisation in $CD4^+$ counts on treatment with a monclonal antibody purified concentrate, there was no difference in the development of AIDS or death, between these patients and those of the control group (Seremitis *et al*, 1993; Goedert *et al*, 1994).

Three studies do not support the view that product purity may influence immune parameters (Mannucci *et al* 1992b; Hilgartner *et al*, 1993; Varon *et al*, 1994). Mannucci *et al* (1992) monitored the $CD4^+$ counts and cell mediated immunity in HIV positive haemophiliacs. One half of the study group were treated with intermediate purity Kryobulin, the remainder being treated with an ion-exchange purified product Beriate P. Over the two year follow up period, both groups demonstrated similar rates of decline in $CD4^+$ counts. The was also no difference in cell mediated immunity between the two groups. A study by Hilgartner *et al* (1993) compared the $CD4^+$ counts in two groups of patients, one group on monoclonally purified factor VIII concentrate, the other on intermediate purity factor VIII concentrate. No significant difference was found between the two groups. A study by Varon *et al* (1994), comparing the effects of a monoclonal antibody purified concentrate to an ion-exchange purified concentrate on $CD4^+$ counts in HIV positive individuals, found no significant benefit on treatment with the monoclonal product.

(ii) Studies in HIV Negative Haemophiliacs

There are fewer reports of comparative studies in HIV negative patients. The available data suggest a continuing decline in $CD4^+$ counts in previously untreated patients, whether they are being treated with intermediate purity (Teitel *et al*, 1989; Cuthbert *et al*, 1990; Fukatake *et al*, 1991) or monoclonally purified products (Fukatake *et al*, 1991), albeit at a slower rate than in HIV infected haemophiliacs.

In contrast to the above, a three year study on previously untreated HIV negative haemophiliac boys (Evans *et al*, 1991) demonstrated that treatment with an intermediate purity factor VIII concentrate 8Y has no effect on $CD4^+$ counts.

In summary, to date studies assessing the effect of factor VIII products of different purity on the rate of $CD4^+$ decline in haemophiliac patients, has resulted only in one small study demonstrating a significant difference between groups of patients on different purity concentrates.

There is no doubt that immunological abnormalities are present in haemophilia patients. It is, however, under debate whether these abnormalities are due to coagulation factor replacement therapy. Since many of these studies failed to take into account the impact viral infections, other than HIV, have on the immune system (Makris *et al*, 1991; Higgins & Goodall, 1991). It has been postulated that infections with agents other than HIV are responsible for the observed immune modulation. This view is supported by the findings of Evans *et al* (1991). Evans and colleagues' study on previously unexposed patients, treated solely with intermediate purity factor VIII concentrate for five years, found no immune abnormalities. These patients also showed no signs of infection with HIV, hepatitis B or hepatitis C.

Cell Type	Immune Abnormality	References
$CD4^+$ lymphocytes	reduced numbers	Ludlam *et al*, 1983. Carr *et al*, 1984. Moffat *et al*, 1985. Antonaci *et al*, 1987. Cuthbert *et al*, 1992.
$CD4^+$ lymphocytes	reduced delayed hypersensitivity responses to antigens	Brettler *et al*, 1986. Madhok *et al*, 1986. Cuthbert *et al*, 1992.
$CD8^+$ lymphocytes	increased numbers	Gamba *et al*, 1987. Freedman *et al*, 1987.

TABLE 1: Summary of the immune abnormalities observed *in vivo,* in haemophiliacs in the absence of HIV infection.

Cell Type	Immune Abnormality	References
T lymphocytes	reduced response to T lymphocyte mitogens	Froebel et al, 1983. Lederman *et al*, 1983. Moffat *et al*, 1985. Mahir *et al*, 1988.
	reduced capacity to secrete IL-2	Madhok *et al*, 1990. Madhok *et al*, 1991.
	reduced capacity to produce IFN-γ	Ruffault *et al*, 1988.
B lymphocytes	hypergammaglobulinaemia	Brevia *et al*, 1985.
monocytes	reduced expression of HLA-DR, LFA-1 and CR3	Roy *et al*, 1988.
	reduced interaction with T lymphocytes	Mannhalter *et al*, 1986.
polymorphonuclear leucoytes	impaired chemotactic, phagocytic and killing abilitites	Antonaci *et al*, 1987.
natural killer cells	impaired response to IFN-β and IFN-γ	Matheson *et al*, 1986.

TABLE 2: Summary of the immune abnormalities observed *in vitro* in cells derived from haemophiliacs receiving coagulation factor replacement therapy.

CHAPTER 2

General Materials and Methods

1. Coagulation Factor Concentrates

A sample of commercial and non-commercial coagulation factor concentrates was selected for the purpose of this study. These samples were of varying purity and processes of manufacture. Tables 3(a) and 3(b) (pages 79 and 80) provide a summary of manufacturing information on these products and a list of their specific activities. The clotting factor activities of all the batches of the products examined are listed in appendix 1 (i), (ii) and (iii). All coagulation factor concentrates were reconstituted according to manufacturers' instructions, and either tested immediately or aliquoted and frozen at -70°C until required.

1.1 Defibrination of Z8 and 8Y

Two of the intermediate factor VIII concentrates (S.N.B.T.S. Z8, and B.P.L 8Y) used in this study were found to be prone to clotting during incubation in assays, for this reason these concentrates were defibrinated prior to use. Concentrates were reconstituted according to manufacturers' instructions and defibrinated using sterile orange sticks following the addition of 2000 U bovine thrombin (Pentex, UK). The sterile orange sticks were used to gently stir the factor VIII solution until all fibrin clots were removed. No other concentrates used in this study required defibrination as all remained clot free throughout the culture period.

2. Cell Culture

2.1 Culture Medium

All cells were cultured in RPMI 1640 medium supplemented with; 100 units/ml penicillin, 100 µg/ml streptomycin and 0.03% L-glutamine. Varying percentages of heat-inactivated foetal calf serum (FCS) were added, depending on cell type. The same batch of FCS was used throughout each series of experiments, unless stated otherwise. All products were supplied by Sigma-Aldrich Company Ltd, UK. All reagents were sterile and sterile handling procedures were applied throughout.

2.2 Culture Conditions

All cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.3 Cryopreservation of Cells

Cryopreservation stocks of cell lines were stored in 10% dimethylsulpoxide in FCS, in liquid nitrogen and could be recovered from this source without difficulty for use in bioassay within a few days.

2.4 Screening for Mycoplasma

All cell lines were screened regularly for mycoplasma contamination using the Gen-Probe Mycoplasma Tissue Culture Rapid Detection System Kit (Gen-Probe Incorporated, San Diego, CA, USA) according to manufacturers instructions.

This kit employs the principle of nucleic acid hybridisation to detect mycoplasma in tissue culture. Using in-solution hybridisation and ribosomal RNA detection, positive samples were detected in three hours. The test kit contained a ^{3}H-labelled DNA probe homologous to *Mycoplasma* and *Acholeplasma* ribosomal RNA. Using this probe all species that commonly infect tissue cultures can be detected.

3. Harvesting Cells and Direct Beta Counting

Cell cultures in 96-well plates were harvested using the Packard Micromate 196 Cell Harvester, onto filtermats (Skatron Instruments Ltd. UK). The filters were dried in an oven before the radioactivity incorporated into cellular DNA was estimated by direct beta counting using a Packard Matrix 96 Direct Beta Counter. The data were then processed using the computer spreadsheet software Quattro Pro.

4. Manipulation of Data

All data from lymphocyte proliferation assays and cytokine bioassays was obtained expressed as cpm from the Direct Beta Counter. The inhibitory percentage of cell proliferation, IL-2 secretion or IL-6 secretion was determined by the following formula:

$$\%\ \text{inhibition of proliferation} = 100\ X \left[1 - \frac{\text{cpm of test - cpm of background}}{\text{cpm of control - cpm of background}} \right]$$
IL-2 or IL-6 secretion

For calculation of percentage inhibition for lymphocyte proliferation assays, cpm of background refers to cells cultured in media alone, and cpm of control to cells stimulated with PHA in the absence of coagulation factor concentrate.

For calculation of percentage inhibition for TGF-β1 bioassays, cpm of background was eliminated from this formula and cpm of control referred to Mv-1-Lu cells cultured in media in the absence of coagulation factor concentrates.

5. Statistical Analysis

Student's *t*-test was used to compare statistically the sample means from two different populations (e.g. viability of PHA-stimulated lymphocytes incubated with coagulation factor concentrates to the viability of the same population of lymphocytes incubated without coagulation factor concentrate).

The strength of association between two quantitative variables was measured by a sample statistic, the correlation coefficient. The correlation coefficient between two independent variables (e.g. percentage inhibition of lymphocyte proliferation by a concentrate and the amount of TGF-β in that concentrate) was calculated using Spearman's rank correlation coefficient.

A p value of <0.05 was considered to be significant.

Product	Manufacturer	Purification	Viral Inactivation	Specific Activity (IU/mg)
High Potency Factor VIII (HPVIII)	Scottish National Blood Transfusion Service, UK.	ion-exchange chromatography	solvent/detergent	>50
Z8	Scottish National Blood Transfusion Service, UK.	conventional fractionation	dry heat (80°C, 72 h)	>2
Alpha VIII	Alpha Therapeutic Corporation, USA.	chemical affinity chromatography	solvent/detergent	>50
Alphanate	Alpha Therapeutic Corporation, USA.	chemical affinity chromatography	solvent/detergent and heat treatment	>1* (>1,000))
Profilate SD	Alpha Therapeutic Corporation, USA.	conventional fractionation	solvent/detergent	>2
Profilate OSD	Alpha Therapeutic Corporation, USA.	conventional fractionation	organic solvent/detergent	>2
8Y	Bio Products Laboratory, UK.	conventional fractionation	dry heat (80°C, 72 h)	>2
8SM	Bio Products Laboratory, UK.	immunoaffinity chromatography	solvent/detergent	5-10* (>3,000)
Immunate	Immuno, Austria.	ion-exchange chromatography	Polyglycate treatment and vapour heating	>50
Haemate P	Behringwerke, Germany.	conventional fractionation	pasteurisation (60°C, 10h)	>2
Monoclate-P	Armour Pharmaceutical Company Ltd, USA.	immunoaffinity chromatography	pasteurisation (60°C, 10h)	5-10* (>3,000)
Kogenate	Bayer plc, UK	gene insertion immunoaffinity chromatography	solvent/detergent	5-10* (>3,000)
Recombinate	Baxter/Hyndland, USA.	gene insertion immunoaffinity chromatography	solvent/detergent	5-10* (>3,000)

TABLE 3(a): Manufacturing information on the factor VIII concentrates used in this study. Concentrates marked with * have human albumin added as a stabiliser, the numbers in the brackets refer to the specific activity before the addition of human albumin.

Product	Manufacturer	Purification	Viral Inactivation	Specific Activity (IU/mg)
Defix	Scottish National Blood Transfusion Service, UK.	ion-exchange chromatography	dry heat (80°C, 72 h)	>2
AlphaNine SD	Alpha Therapeutic Corporation, USA.	ion-exchange chromatography	solvent/detergent	>50
9MC	Bio Products Laboratory, UK.	metal chelate affinity chromatography	solvent/detergent	>100
9A	Bio Products Laboratory, UK.	ion-exchange chromatography	dry heat (80°C, 72 h)	>2
Mononine	Armour Pharmaceutical Company, USA.	immunoaffinity chromatography	sodium thiocyanate and ultrafiltration	>150
Concentré De Facteur Willebrand#	Bio-Transfusion, Lille, UK	ion-exchange chromatography	solvent/detergent	>100

TABLE 3(b): Manufacturing information on the factor IX and von Willebrand factor concentrates used in this study. The product marked # is a von Willebrand factor concentrate. All others are factor IX concentrates.

CHAPTER 3

Effects of Coagulation Factor Concentrates on Normal Lymphocytes

INTRODUCTION

It is now recognised that patients receiving coagulation factor replacement therapy may have abnormalities of the immune system, irrespective of HIV status. These abnormalities have been observed both *in vivo* and *ex vivo*. It has been proposed that these disturbances are caused either by the massive protein load, specific impurities or the effect of viruses in coagulation factor concentrates.

Efforts to identify contaminants responsible have involved testing coagulation factor concentrates, and manipulations of these concentrates in various *in vitro* assay systems which assess immunomodulating activity. One such assay commonly used for this purpose is the PHA-stimulated lymphocyte proliferation assay (Froebel *et al*, 1983; McDonald *et al*, 1985; Lederman *et al*, 1986; Hay & McEvoy, 1989; Vermot-Desroches *et al*, 1992). PHA is a T cell stimulator which causes non-dividing lymphocytes to proliferate and differentiate, so that they display some of the same functional characteristics as antigen activated lymphocytes (Sharon, N. 1983). For this reason, mitogenic stimulation of lymphocytes with PHA is commonly used to detect immune modulating activity. At the outset of this study limited data were available on the effects of factor VIII, factor IX and von Willebrand factor concentrates prepared by ion-exchange, affinity, immunoaffinity and recombinant techniques in this assay. Hence, a wide range of commercial and non-commercial coagulation factor concentrates of varying purity were selected to be assayed for their immune modulating activity by PHA-stimulated lymphocyte proliferation assay.

Even though many researchers have used lymphocyte proliferation assays to assess the immunomodulatory activity of concentrates, the reported concentration of PHA used for stimulation of proliferation and the length of incubation before harvest varies greatly. Experimental conditions can often affect the outcome of experiments as exemplified by the data obtained from mixed lymphocyte reactions. One group reporting enhanced lymphocyte proliferation three others reporting inhibition of proliferation (Lederman *et al*, 1986; Schrieber *et al*, 1987; Hay & McEvoy, 1989; Batchelor *et al*, 1992). Since, PHA concentration and incubation time are both

important factors whose variability can alter the results significantly, great care was taken to optimise the assay before testing the concentrates.

Another widely used method of assessing the immune modulation of coagulation factor concentrates, is the study of the effects coagulation factor concentrates have on IL-2 secretion by PHA-stimulated T cells (Lederman *et al*, 1986; Thorpe *et al*, 1989; Wadhwa *et al*, 1992; Wadhwa *et al*, 1994). At the outset of this study the data available on the effects of factor VIII concentrates of varying purity on IL-2 secretion was much more complete than that from lymphocyte proliferation studies. The results demonstrated that some, but not all, intermediate purity factor VIII concentrates and those purified by ion-exchange chromatography inhibited IL-2 secretion, whereas monoclonal antibody purified and recombinant products did not. However, no study had directly compared the effects of a sample of coagulation factor concentrates on both PHA-stimulated lymphocyte proliferation and IL-2 secretion. Therefore, in order to present a full set of data on the immune modulating effects of coagulation factor concentrates chosen for this study, it was necessary to test for their effects on IL-2 secretion by PHA-stimulated T cells as well as for their effects on proliferation.

All publications relating to the effects of factor VIII concentrates on IL-2 secretion use almost identical experimental conditions. For this reason, I have used the same set of experimental conditions as published thereby allowing comparison between IL-2 secretion data on my sample of concentrates and that reported in the literature.

Having established which coagulation factor concentrates from my chosen sample have the ability to inhibit lymphocyte function, a brief study of the mechanism of inhibition was considered appropriate. Direct cytotoxicity of intermediate purity and ion-exchange purified concentrates on PHA-stimulated lymphocytes had been examined previously (McDonald *et al*, 1985; Lederman *et al*, 1986; Wang *et al*, 1986; Vermot-Desroches *et al*, 1992), and concentrates of both levels of purity found not to mediate their effects on lymphocyte proliferation by cytotoxicity. However, the effects of other inhibitory coagulation factor concentrates, or those purified by affinity techniques had not been examined. Therefore, in order to establish whether these concentrates inhibited lymphocyte proliferation by

cytotoxicity, the viability of PHA-stimulated lymphocytes incubated in the presence and absence of coagulation factor concentrate was estimated by nigrosin dye exclusion experiments.

The cytokine IL-6 is also expressed by lymphocytes and its production up-regulated by numerous signals including mitogenic stimulation and the cytokine IL-2 (Hirano, 1994). IL-6 has several important immunological functions, including effects on T cell activation, growth, differentiation and IL-2 secretion (Lotz *et al*, 1988; Tosato & Pike, 1988; Uttenhove *et al*, 1988). Since, IL-6 secretion by lymphocytes is up-regulated by IL-2, a fall in IL-6 production by lymphocytes incubated with an inhibitory concentrate would be expected. For this reason the effects that the sample of coagulation factor concentrates have on the secretion of IL-6 by lymphocytes was also examined.

The information obtained from the above experiments has provided a detailed study on the immune modulating effects of coagulation factor concentrates of varying purity. In addition, these experiments have allowed the comparison of the effects that coagulation factor concentrates have on two commonly used assay systems, lymphocyte proliferation and lymphocyte IL-2 secretion. Finally, further insight into the mechanism of inhibition by some concentrates was obtained.

MATERIALS AND METHODS

1. Cells

1.1. Separation of Peripheral Blood Mononuclear Cells from Whole Blood

Peripheral blood mononuclear cells (PBMC) were separated from fresh heparinised venous blood or citrated buffy coats (Scottish Blood Transfusion Service {S.N.B.T.S.}, Edinburgh, UK) collected from donations of healthy human blood. Heparinised blood samples were diluted 1:2 and citrated buffy coats diluted 1:4 with RPMI 1640. PBMC were isolated by centrifugation over Lymphoprep (Density 1.077 g/ml. Nycomed Ltd, UK) at 800g for 15 minutes. The cells at the interface were removed and washed twice in RPMI 1640 medium containing 10% FCS. The mononuclear cells were subjected to slow-speed centrifuge runs at 150g for 10 minutes to separate from contaminating platelets, then used immediately.

1.2 Origin and Maintenance of Cell Lines

(i) CTLL Cells

The IL-2 dependent murine T cell line, CTLL was obtained from the European Collection of Animal Cell Cultures (ECACC No: 87031904. Porton Down, Salisbury, UK) for use in IL-2 bioassays. Cells were maintained at a density of 3-9x10^4 cell/ml, in 25 cm^2 culture flasks in RPMI 1640 medium supplemented with 10% FCS, 20% human PBMC conditioned medium and 20 IU/ml recombinant human IL-2 (Sigma-Aldrich Ltd.). Cultures were maintained in a three day feeding schedule.

(ii) B9 Cells

The IL-6 dependent murine hybridoma B cell line, B9, were obtained from D. Hutchins (MRC, Edinburgh) for use in IL-6 bioassays. Cells were maintained in 25 cm^2 culture flasks on RPMI 1640 medium containing 10% FCS plus a 1/100 dilution of human PBMC conditioned medium. Cultures were maintained on a three day feeding schedule.

1.3. Preparation of Human PBMC Conditioned Medium

Conditioned medium required for maintenance of the cell lines CTLL and B9 was prepared by isolating PBMC from several human buffy coats and culturing them at a concentration of $5x10^6$ cells/ml in RPMI 1640 medium supplemented with 5% FCS and 90 μg/ml PHA (Reagent grade. Murex Diagnostics Ltd, UK) (Grimm & Rosenberg, 1982). Cultures were incubated for 40 hours before supernatants were harvested by centrifugation at 150g for 15 minutes, then at 1500g for 15 minutes. The resulting conditioned medium from the different buffy coats was combined, sterile filtered and frozen at -70°C until required.

2. Lymphocyte Proliferation Assays

2.1 Optimisation of Lymphocyte Proliferation Assay

Proliferative responses of PBMC to various concentrations of PHA were measured by culturing PBMC in 96-well flat bottomed microtitre plates (Falcon Microtest III. A & J Beveridge Ltd. UK) with increasing concentrations of PHA. For this purpose, serial doubling dilutions of PHA in culture medium (0.7-720 μg/ml) were prepared in triplicate, in 100 μl volumes. To each PHA dilution, coagulation factor concentrate was added at a final concentration of 2 IU/ml. A PBMC suspension containing $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FCS was prepared and 100 μl added to each well. Control cultures of PBMC alone and PBMC with dilutions of PHA, in the absence of coagulation concentrate, were included. Cultures were incubated for 96 hours before harvesting, as described elsewhere (page 77). Proliferation of cells in response to PHA was measured by pulsing cultures with 1μCi/well of [methyl-^{3}H]thymidine (Amersham Life Science, UK) 18 hours before cell harvesting.

Having established the optimal PHA concentration for examining the effects of coagulation factor concentrates on lymphocyte proliferation the optimal length of culture was determined. For this purpose, six identical plates containing 100 μl PBMC suspension, as detailed above, were stimulated with the optimal concentration of PHA (1 μg/ml) and incubated with 100 μl of increasing concentrations of a factor VIII concentrate (0.5-2 IU/ml) prepared in culture medium. Plates were harvested on consecutive days as described elsewhere (page 77). Proliferation of cells in response to PHA was measured by pulsing cultures with 1μCi/well of [methyl-^{3}H]thymidine (Amersham Life Science, UK) 18 hours before cell harvesting.

2.2 Optimised PHA Stimulated Lymphocyte Proliferation Assay

Proliferative responses to 1 μg/ml PHA were measured by culturing PBMC in 96-well flat-bottomed microtitre plates. For this purpose, a cell suspension of $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FCS was prepared. Triplicate wells containing 100 μl of cell suspension and 100 μl of serial doubling dilutions of coagulation factor concentrates (0.004-2 IU/ml) were prepared in culture medium. The levels of coagulation factor concentrates used for assay purposes reflect levels readily achievable *in vivo* during replacement therapy (Batchelor *et al*, 1992).

Control cultures of PBMC in culture medium alone, PBMC and PHA alone, and PBMC plus coagulation factor concentrate in the absence of PHA, were included in each assay. Cultures were incubated for 96 hours before harvesting. Proliferation of cells in response to PHA was measured by pulsing cultures with 1μCi/well of [methyl-^{3}H]thymidine 18 hours before cell harvesting.

In order to overcome the variability of donor responses to mitogen, each batch of coagulation factor concentrate was tested three times on PBMC suspensions prepared from three individuals.

In some assays heparin (Leo Laboratories Ltd, UK) was included in the culture medium at a final concentration of 1 IU/ml. Exogenous IL-2 (recombinant. Sigma-Aldrich Company Ltd.) was also added to some PHA-stimulated lymphocyte proliferation assays at a final concentration of 10 IU/ml or 100 IU/ml.

2.3 Cell Viability

The effects of coagulation factor concentrates on the viability of PHA-stimulated lymphocytes was investigated by nigrosin dye exclusion. Triplicate wells containing 1 ml of a cell suspension of $1x10^6$ cells/ml in RPMI 1640 media supplemented with 10% FCS were prepared and incubated with or without 1 IU/ml of coagulation factor concentrates. PHA was included at a final concentration of 1 μg/ml.

Viability was estimated at 24 hour intervals by counting 200 cells four times and calculating the mean percentage nigrosin positive cells. Cell viabilty of cells in the presence or absence of coagulation factor concentrates was compared using a paired *t*-test.

3. Cytokine Bioassays

3.1 Stimulation of Interleukin 2 Secretion

For stimulation of IL-2 secretion, PBMC were washed in RPMI 1640 medium and then resuspended at $2x10^6$ cells /ml in RPMI 1640 medium containing 10% FCS and 90 µg/ml PHA (Thorpe *et al*, 1989). To investigate the effects of clotting factor concentrates on IL-2 secretion, 1 ml aliquots of cell suspension were incubated with or without 1 IU/ml of clotting factor concentrate in 12-well flat bottomed microtitre plates (Costar Ltd.). Control cultures of PBMC in culture medium alone, PBMC and PHA alone, and PBMC plus coagulation factor concentrate in the absence of PHA were included in each assay. Supernatants were harvested by centrifugation after 48 hours and stored at -20°C until assayed for IL-2.

3.2 Interleukin 2 Bioassay

IL-2 was measured using a bioassay based on the mouse T cell line, CTLL's dependence on IL-2 (Gillis *et al*, 1987; Wadhwa *et al*, 1991). CTLL cultures were fed, as previously described, 48 hours prior to inclusion in the IL-2 bioassay. For assay CTLL cells were washed twice in PBS and resuspended at a concentration of $1x10^5$ cells/ml in RPMI 1640 medium supplemented with 10% FCS. Triplicate wells, in 96-well flat-bottomed plates were set up containing 50 µl cell suspension and either 50 µl of test supernatant or 50 µl of a titration of a standard recombinant IL-2 (Sigma-Aldrich Ltd.). Control cultures of CTLL cells in the presence of 1 IU/ml of coagulation factor were included in each assay. Cultures were incubated for 18 hours, then pulsed for 4 hours with 0.5 µCi/ well [methyl-^{3}H]thymidine prior

to harvesting. A standard curve of counts per minute (cpm) versus concentration of IL-2 was plotted and activity of unknown samples determined by comparing test results with the standard curve.

3.3 Stimulation of Interleukin 6 Secretion

For stimulation of IL-6 secretion, PBMC were washed in RPMI 1640 medium and then resuspended at $1x10^6$ cells/ml in RPMI 1640 medium containing 10% FCS and 90 μg/ml PHA. To investigate the effects of clotting factor concentrates on IL-6 secretion, 1 ml aliquots of cell suspension were incubated with or without 1 IU/ml of clotting factor concentrate in 12-well flat bottomed microtitre plates. Control cultures of PBMC in culture medium alone, PBMC and PHA alone, and PBMC plus coagulation factor concentrate in the absence of PHA were included in each assay. Supernatants were harvested by centrifugation after 24 hours and stored at -20°C until assayed for IL-6.

3.4 Interleukin 6 Bioassay

IL-6 bioactivity was measured using a [methyl-^{3}H]thymidine incorporation assay based on the growth response of the IL-6 dependent cell line B9 (Hutchins *et al*, 1990). B9 cells were fed as previously described 48 hours prior to inclusion in the IL-6 bioassay. Cells were washed in RPMI 1640 medium to remove residual growth factor and resusupended at a concentration of $4x10^4$ cells/ml in RPMI 1640 medium supplemented with 10% FCS. Triplicate wells, in 96-well flat-bottomed plates were set up containing 100 μl aliquots of cell suspension and either 100 μl of test supernatant containing IL-6 or 100 μl of a titration of a standard recombinant IL-6 (Sigma-Aldrich Ltd.). Control cultures of B9 cells in the presence of 1 IU/ml of coagulation factor concentrates were included in each assay. Cultures were

incubated for three days and pulsed for the final five hours of culture with 1μCi of [methyl-^{3}H]thymidine prior to harvesting. A standard curve of cpm versus concentration of IL-6 was plotted and activity of unknown samples determined by comparing test results with the standard curve.

RESULTS

1. Effects of Coagulation Factor Concentrates on Lymphocyte Proliferation Following Stimulation with PHA.

1.1 Optimisation of Lymphocyte Proliferation Assay

Many researchers have studied the effects of coagulation factor concentrates on PHA stimulated lymphocyte proliferation. However, the PHA concentration used to stimulate the cells has varied greatly. To determine the optimal concentration of PHA for stimulation of proliferation, lymphocytes were incubated with increasing concentrations of PHA (0.7-720 μg/ml). A semilog graph was constructed of concentration of PHA (logarithmic) versus cpm (linear), Figure 5. Optimal proliferation was observed with 22.5 and 45 μg/ml PHA. The effects of 2 IU/ml of two factor VIII concentrates were tested at each concentration of PHA, enabling a concentration of PHA to be determined at which coagulation factor concentrates caused the greatest inhibition. The intermediate purity concentrate 8Y was found to inhibit proliferation, the immunoaffinity purified concentrate Monoclate P did not. Inhibition of proliferation by the intermediate purity concentrate was observed to be at its greatest at 0.7 to 5.6 μg/ml of PHA. A final concentration of 1 μg/ml of PHA was selected for use in lymphocyte proliferation assays, since it lay within the optimal range and had been used previously by some researchers (McDonald *et al*, 1985; Vermot-Desroches *et al*, 1992).

As with the PHA concentration used to stimulate the cells, the reported time of harvesting of proliferation assays also varies. To determine the optimal time to harvest the lymphocyte proliferation assays, identical plates containing lymphocytes stimulated with 1 μg/ml PHA, and increasing concentrations of factor VIII concentrate (0.5 -2 IU/ml), were harvested on consecutive days, Figure 6.

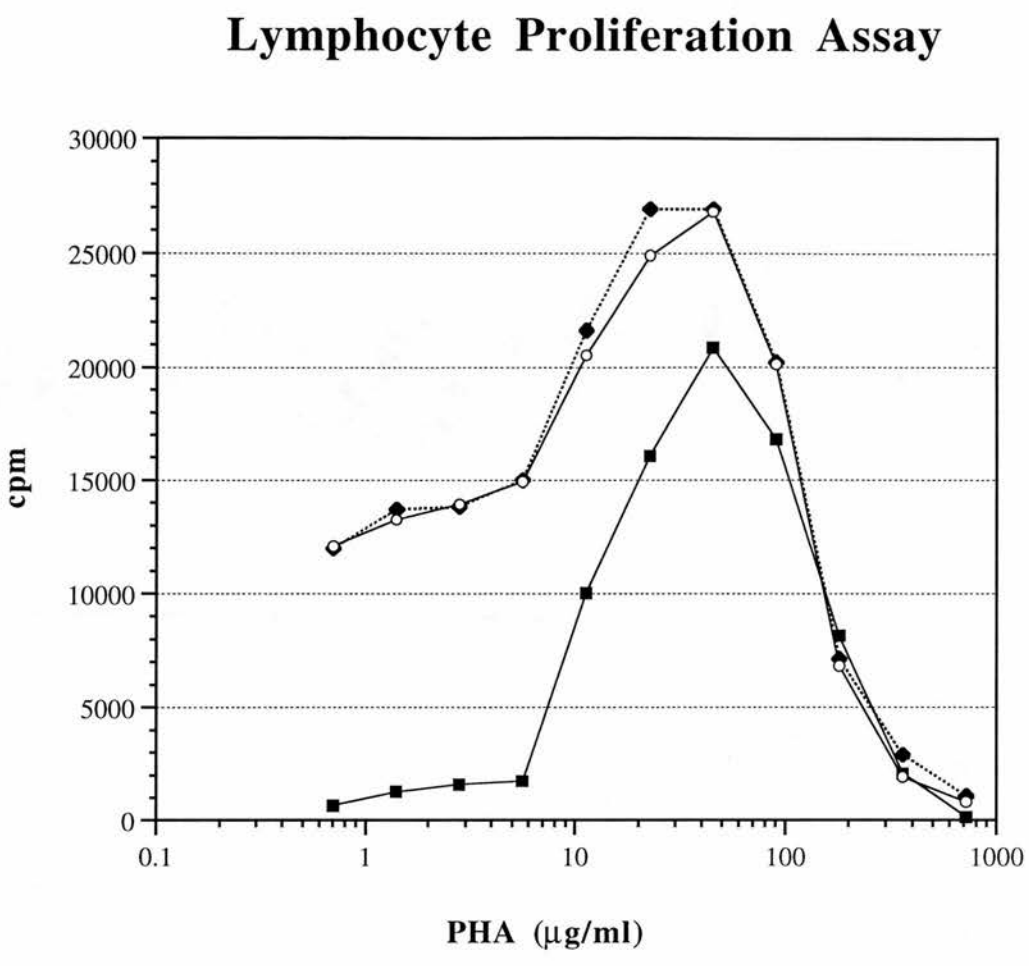


FIGURE 5: Dose response of lymphocytes to the T cell mitogen PHA, and the effect 2 IU/ml of two factor VIII concentrates have on lymphocyte proliferation stimulated by different concentrations of PHA.

—○— PHA alone

·····◆····· Monoclate-P (immunoaffinity purified)

—■— 8Y (intermediate purity)

Lymphocyte Proliferation Assay

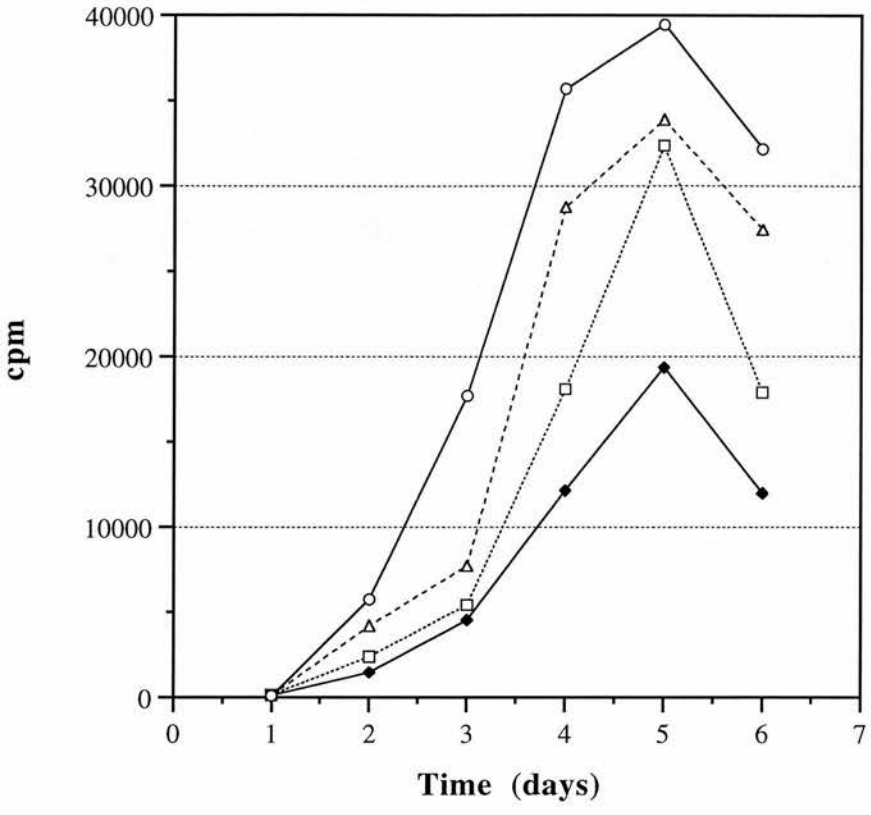


FIGURE 6: Inhibition of proliferation of PHA-stimulated lymphocytes by a factor VIII concentrate. The peak response of lymphocytes to 1 µg/ml PHA occured on day 5. Proliferation was routinely assayed for on day 4 and found to be adequate.

—○— PBMC + PHA alone

----△---- PBMC + PHA + 0.5 IU/ml factor VIII concentrate

·····□····· PBMC + PHA + 1 IU/ml factor VIII concentrate

—◆— PBMC + PHA + 2 IU/ml factor VIII concentrate

The peak response of lymphocytes to 1 μg/ml PHA occurred on day 5. However, proliferation was routinely assayed on day 4 and found to be adequate.

This preliminary experiment demonstrates that inhibition of lymphocyte proliferation by this factor VIII concentrate occurs in a dose-dependent manner.

1.2 PHA-Stimulated Lymphocyte Proliferation Assay

Following optimisation of culture conditions, the sample of commercial and non-commercial coagulation factor concentrates (concentrates of factors VIII, IX and von Willebrand factor) were examined for their ability to inhibit PHA-stimulated lymphocyte proliferation. Concentrates were tested in triplicate over a range of concentrations (0.004-2 IU/ml), which are readily achievable during replacement therapy.

Results obtained from lymphocyte proliferation assays were expressed in cpm. The average cpm of each dilution triplicate was calculated and converted to percentage inhibition of proliferation using the formula on page 73. Since, each batch of concentrate was tested in three separate lymphocyte proliferation assays, with lymphocytes from three different donors, an average percentage inhibition was calculated for each dilution point. Once the results for each batch were compiled, semilogarithmic graphs were plotted, displaying log concentration of coagulation factor concentrate (IU/ml) versus percentage inhibition of lymphocyte proliferation, Figures 7-24.

More than one batch of concentrate was tested, where possible. In these cases the average percentage inhibition at each coagulation factor dilution point for the batches was calculated and plotted against the log concentration of coagulation factor concentrate (IU/ml). The standard deviation between batches is represented on the graphs as error bars.

All coagulation factor concentrates which inhibited PHA-stimulated lymphocyte proliferation did so in a dose-dependent manner.

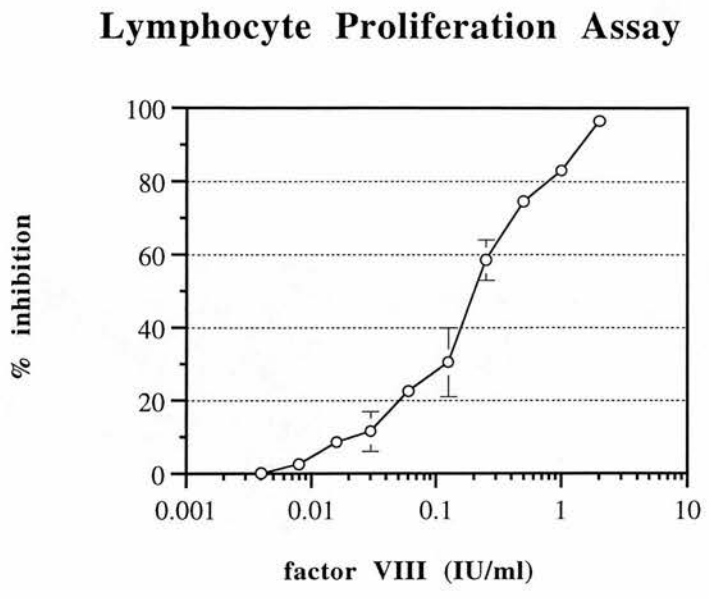


FIGURE 7: Inhibition of proliferation of PHA-stimulated lymphocytes by two batches of the intermediate purity factor VIII concentrate Z8.

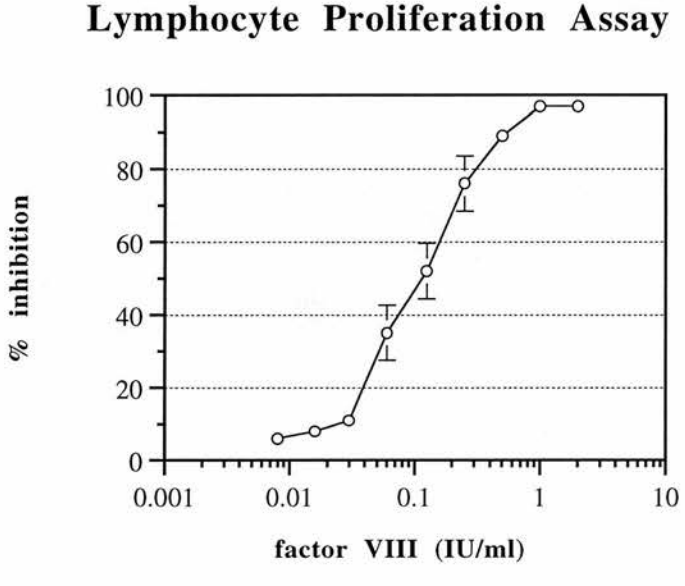


FIGURE 8: Inhibition of proliferation of PHA-stimulated lymphocytes by three batches of the intermediate purity factor VIII concentrate Profilate SD.

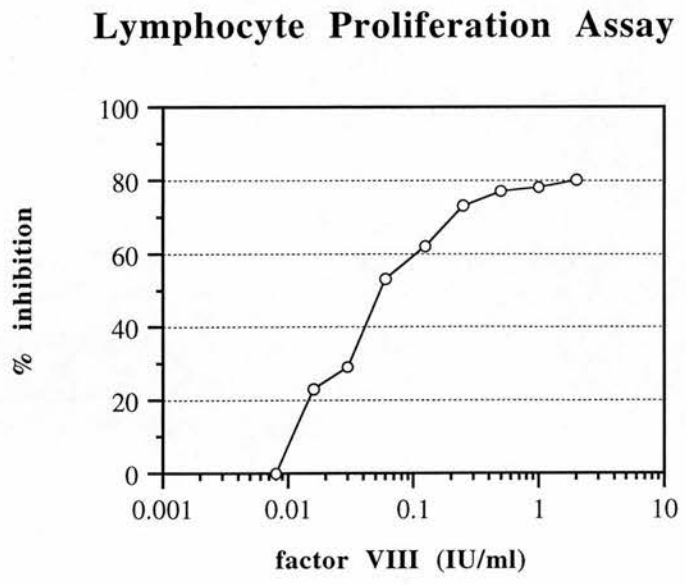


FIGURE 9: Inhibition of proliferation of PHA-stimulated lymphocytes by a batch of the intermediate purity factor VIII concentrate Profilate OSD.

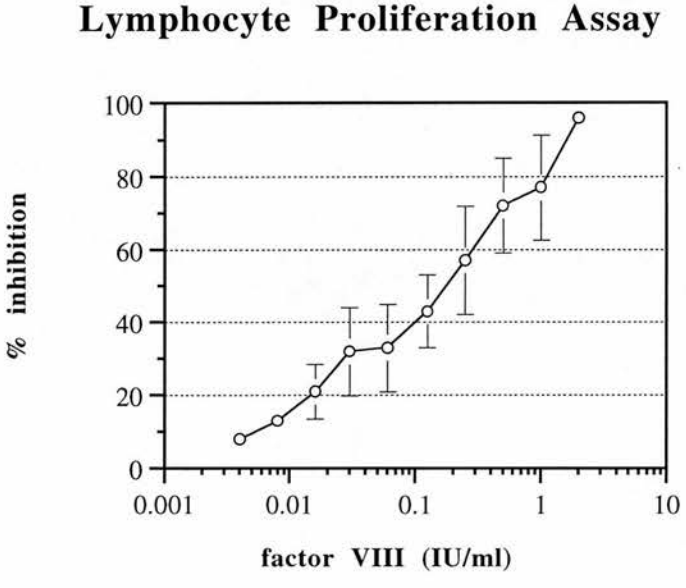


FIGURE 10: Inhibition of proliferation of PHA-stimulated lymphocytes by three batches of the intermediate purity factor VIII concentrate 8Y.

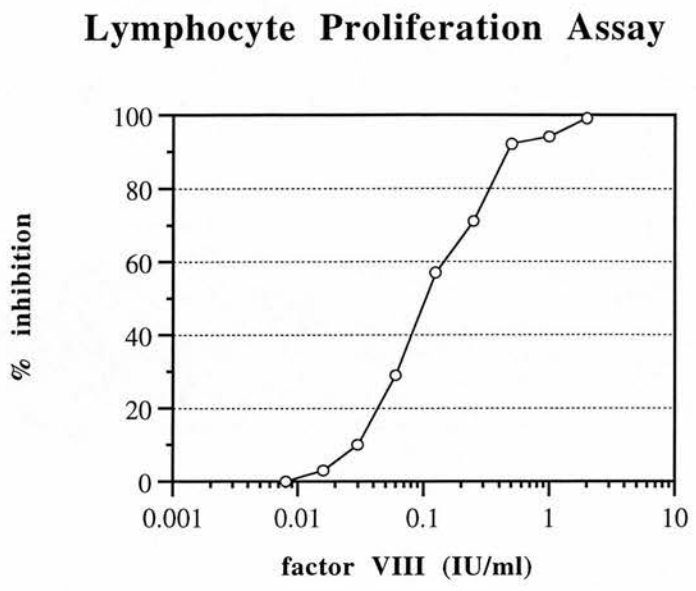


FIGURE 11: Inhibition of proliferation of PHA-stimulated lymphocytes by one batch of the intermediate purity factor VIII concentrate Haemate P.

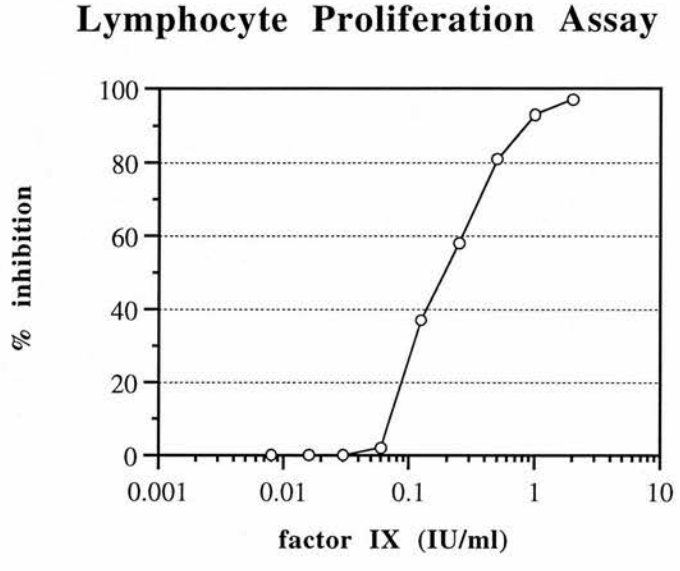


FIGURE 12: Inhibition of proliferation of PHA-stimulated lymphocytes by one batch of the intermediate purity factor IX concentrate Defix.

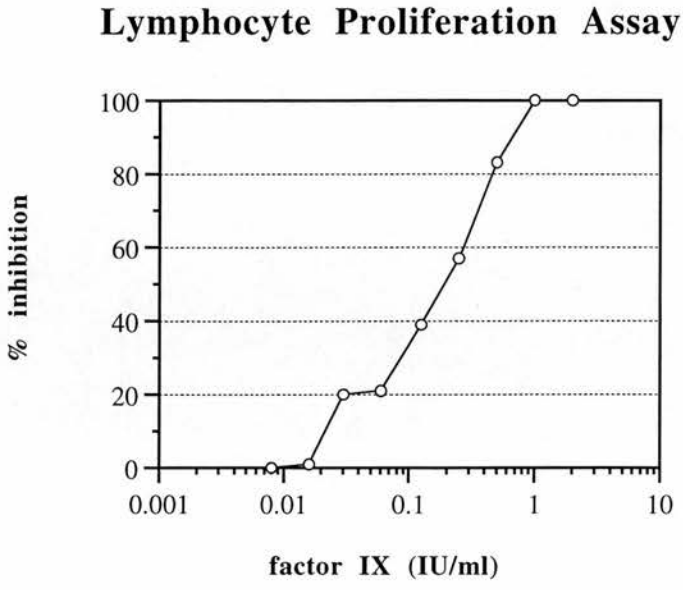


FIGURE 13: Inhibition of proliferation of PHA-stimulated lymphocytes by one batch of the ion-exchange purified factor IX concentrate 9A.

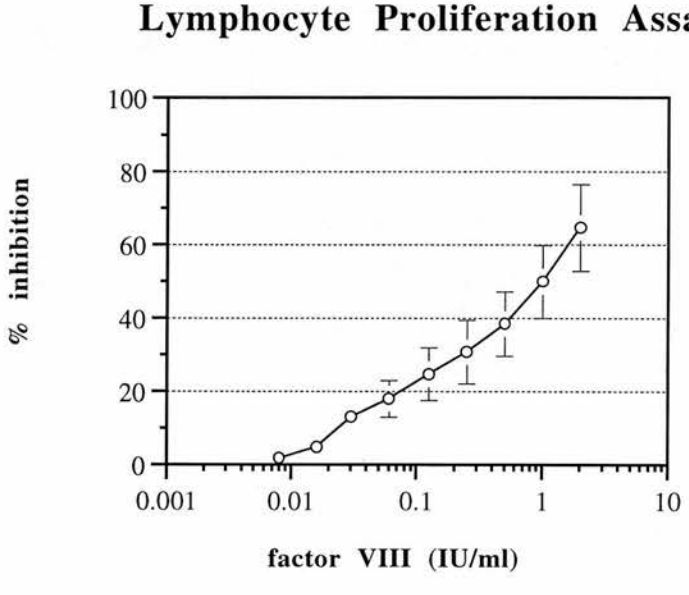


FIGURE 14: Inhibition of proliferation of PHA-stimulated lymphocytes by six batches of the ion-exchange purified factor VIII concentrate High Potency Factor VIII (HPVIII).

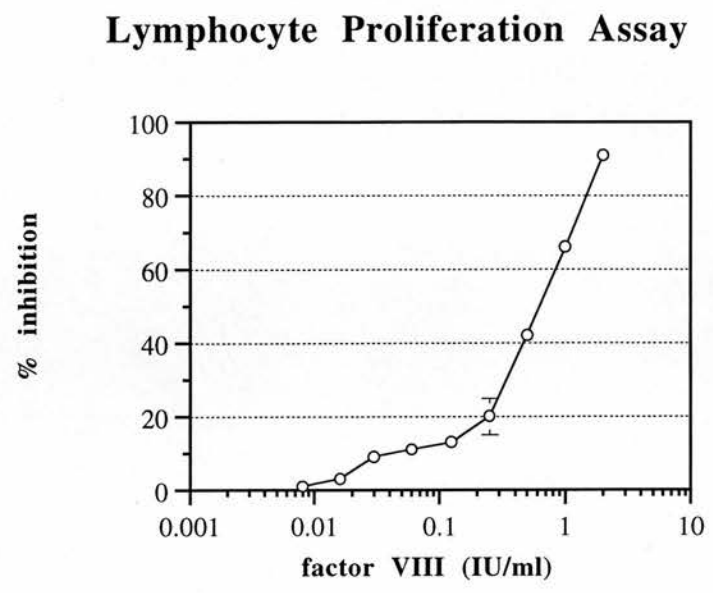


FIGURE 15: Inhibition of proliferation of PHA-stimulated lymphocytes by five batches of the ion-exchange purified factor VIII concentrate Immunate. (Error bars too close to points to be illustrated.)

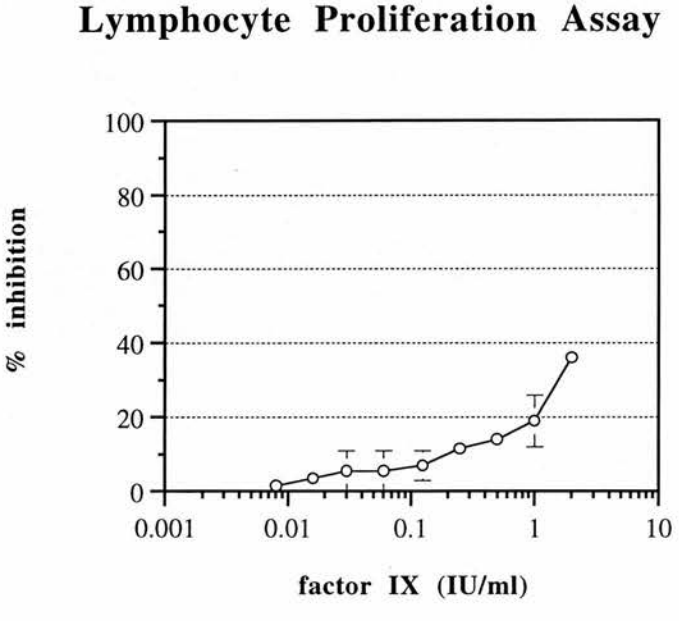


FIGURE 16: Inhibition of proliferation of PHA-stimulated lymphocytes by two batches of the ion-exchange purified factor IX concentrate AlphaNine SD.

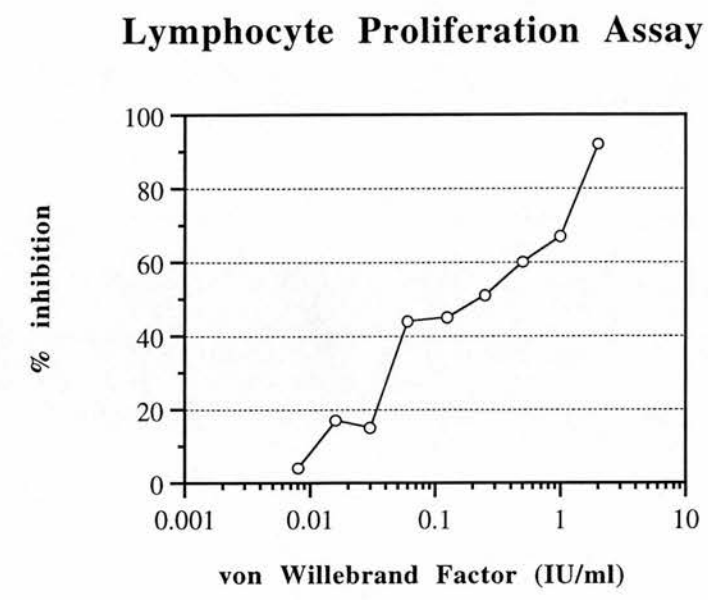


FIGURE 17: Inhibition of proliferation of PHA-stimulated lymphocytes by one batch of the ion-exchange purified von Willebrand factor concentrate Concentre De Facteur Willebrand.

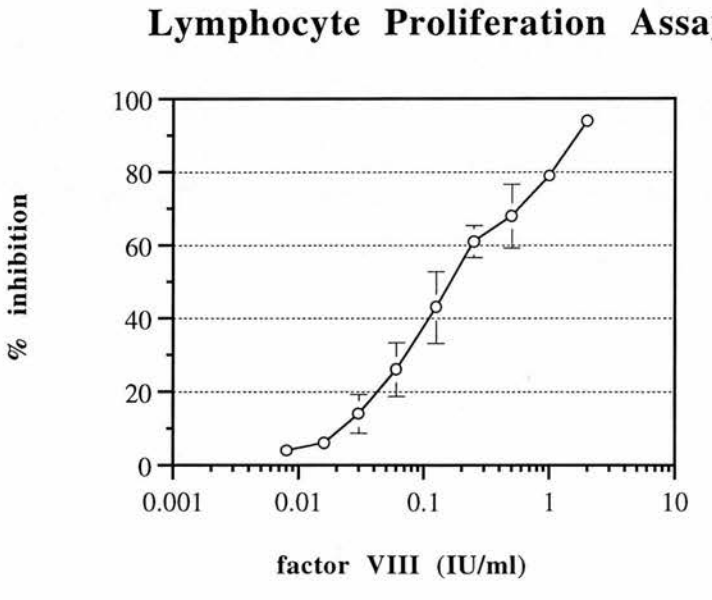


FIGURE 18: Inhibition of proliferation of PHA-stimulated lymphocytes by three batches of the affinity purified factor VIII concentrate Alpha VIII.

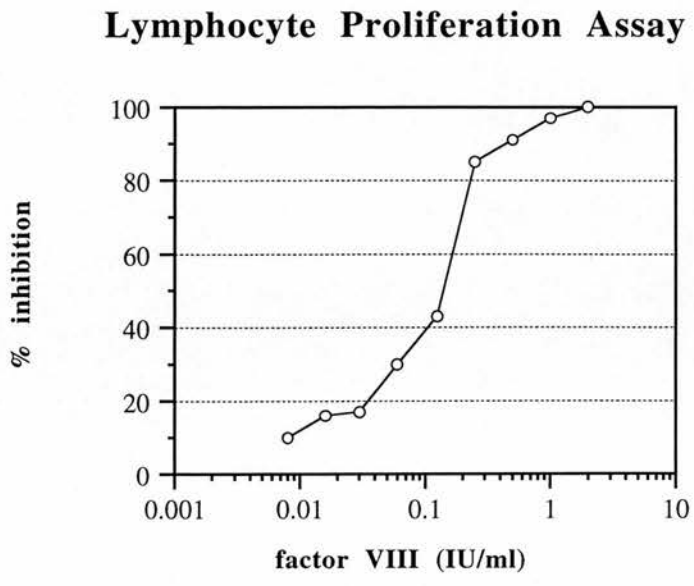


FIGURE 19: Inhibition of proliferation of PHA-stimulated lymphocytes by one batch of the affinity purified factor VIII concentrate Alphanate.

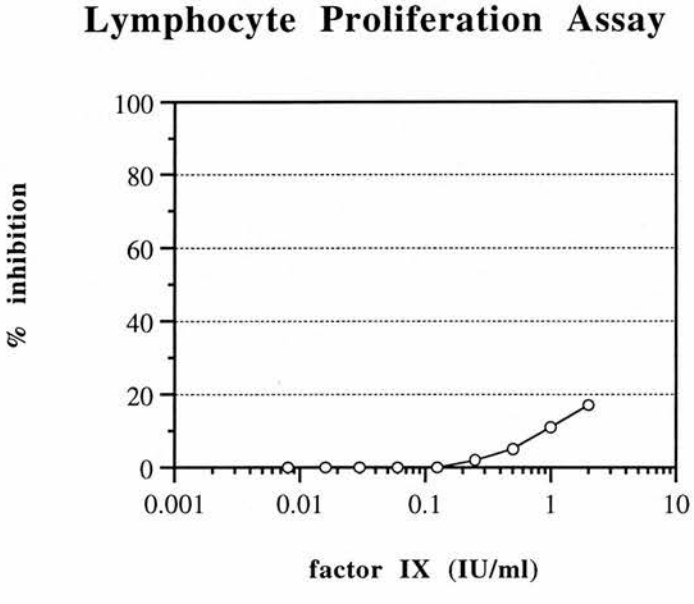


FIGURE 20: Slight inhibition of proliferation PHA-stimulated lymphocytes by one batch of affinity purified factor IX concentrate 9MC.

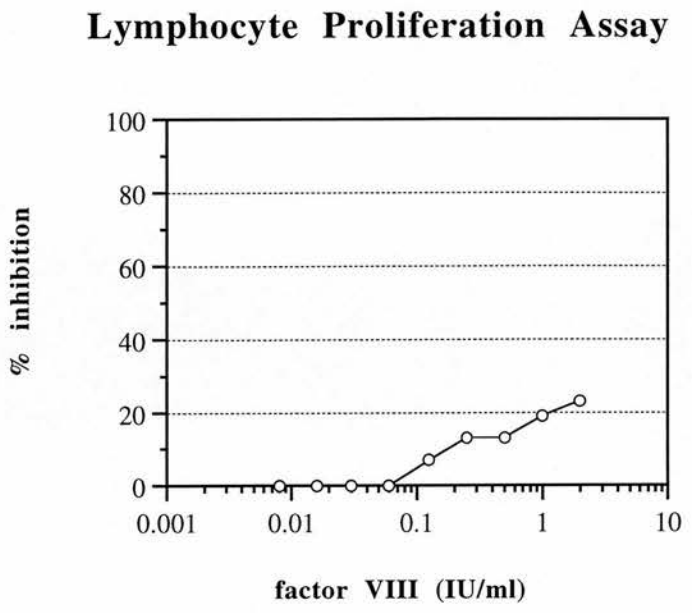


FIGURE 21: Slight inhibition of proliferation of PHA-stimulated lymphocytes by one batch of the immunoaffinity purified factor VIII concentrate 8SM.

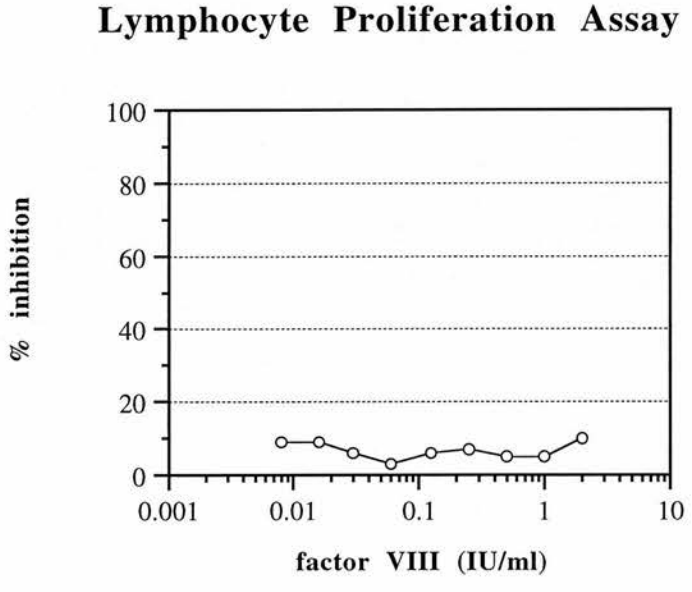


FIGURE 22: Absence of inhibition of proliferation of PHA-stimulated lymphocytes by one batch of the immunoaffinity purified factor VIII concentrate Monoclate P.

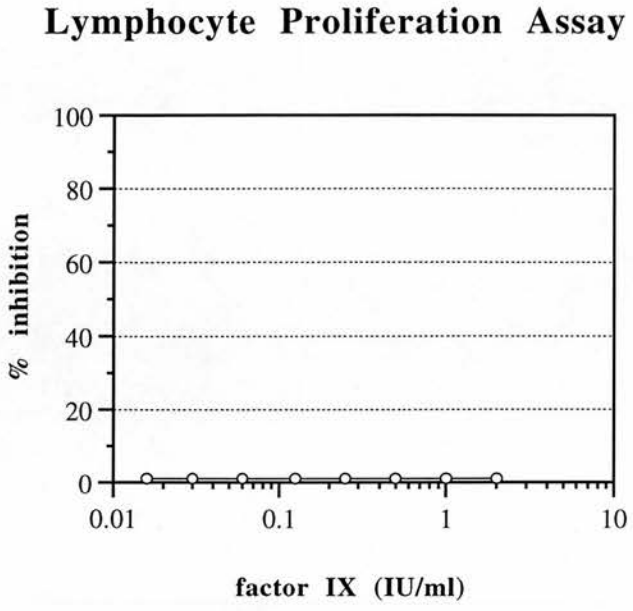


FIGURE 23: Absence of inhibtion of proliferation of PHA-stimulated lymphocytes by one batch of the immunoaffintiy purified factor IX concentrate Mononine.

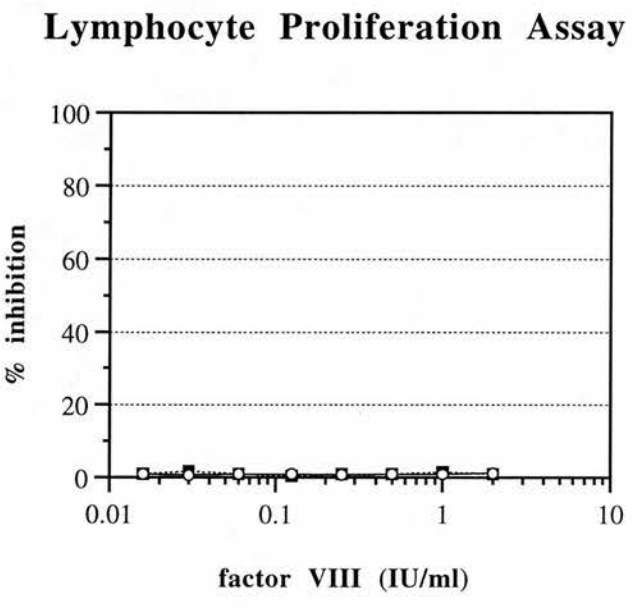


FIGURE 24: Absence of inhibition of PHA-stimualted lymphocyte proliferation by two recombinant factor VIII products Kogenate and Recombinate.

—○— Kogenate ·····■····· Recombinate

The percentage inhibition of proliferation of PHA-stimulated lymphocytes achieved by 1 IU/ml of each batch of concentrate is summarised in Tables 4(a), (b) and (c). All intermediate purity coagulation factor concentrates caused almost total inhibition of lymphocyte proliferation, Table 4(a), Figures 7-13. One exception was a batch of the factor VIII concentrate 8Y, manufactured by BPL. This particular batch inhibited PBMC proliferation by only 49% when present at this concentration.

All ion-exchange purified coagulation factor concentrates, when present at a concentration of 1 IU/ml, inhibited PBMC proliferation to some extent, Table 4(b), Figures 14-17. However, one product in this category, AlphaNine SD, possessed significantly less inhibitory activity than other products of the same group, Figure 16.

All affinity purified products also inhibited proliferation of PHA-stimulated lymphocytes to some extent, Table 4(c), Figures 18-20. However, the factor IX concentrate 9MC, purified by metal chelate affinity was less inhibitory than the other affinity purified products Alphanate and Alpha VIII, both factor VIII concentrates purified by heparin affinity.

The immunoaffinity purified and recombinant coagulation factor concentrates, when present at 1 IU/ml, in general had no effect on lymphocyte proliferation, Table 4(c), Figures 21-24. The only exception being the factor VIII concentrate 8SM, which caused 19% inhibition of proliferation.

Concentrates, in general, exhibited little batch to batch variation. For instance, six batches of Immunate gave inhibitory activities ranging from 57% to 70% (mean 66.4%). However, the factor VIII concentrate HPVIII manufactured by S.N.B.T.S. showed great batch to batch variation, from 27% to 70% (mean 47.5%).

Purification	Product	Batch Number	% Inhibition of Lymphocyte Proliferation
Intermediate	Z8	1-369	82
	Z8	1-378	84
	Profilate SD	AR2003A	99
	Profilate SD	AR2004A	94
	Profilate SD	AR2008A	97
	Profilate OSD	AR4205A	77
	8Y	FHC 0484	85
	8Y	FHC 0494	49
	8Y	FHC 4133	97
	Haemate P	978641	94
	Defix*	20720	93
	9A*	FJA 0114	100
Range			**49 - 100**

TABLE 4(a):- Summary of lymphocyte proliferation assay results for intermediate purity products.

Percentage inhibition of lymphocyte proliferation represents values obtained at 1 IU/ml of coagulation factor concentrate. Products marked with * are factor IX concentrates. All others are factor VIII concentrates.

Purification	Product	Batch Number	% Inhibition of Lymphocyte Proliferation
Ion-exchange	HPVIII	10040	53
	HPVIII	20390	37
	HPVIII	30540	70
	HPVIII	30560	27
	HPVIII	30650	67
	HPVIII	40820	31
	Immunate	09H309210S	70
	Immunate	09H319210S	68
	Immunate	09H319301S	69
	Immunate	09H349211S	68
	Immunate	09H359212S	57
	AlphaNine SD*	CA21401A	12
	AlphaNine SD*	CA2403AB	26
	Concentré De Facteur Willebrand#	87920070	67
Range			12 - 70

TABLE 4(b):- Summary of lymphocyte proliferation assay results for ion-exchange products.

Percentage inhibition of lymphocyte proliferation represents values obtained at 1 IU/ml of coagulation factor concentrate. Products marked with * are factor IX concentrates, that marked with # is a von Willebrand factor concentrate. All others are factor VIII concentrates.

Purification	Product	Batch Number	% Inhibition of Lymphocyte Proliferation
Affinity	Alpha VIII	AP2005A	79
	Alpha VIII	AP2006A	76
	Alpha VIII	AP2008A	83
	Alphanate	AS4701A	97
	9MC*	PMC 2203	17
Immunoaffinity	8 SM	FHD 4143	19
	Monoclate P	J53706	5
	Mononine*	J82612	0
Recombinant	Kogenate	ADAH1	0
	Recombinate	95F22A250	0

TABLE 4(c):- Summary of lymphocyte proliferation assay results for affinity, immunoaffinity purified and recombinant products.

Percentage inhibition of lymphocyte proliferation represents values obtained at 1 IU/ml of coagulation factor concentrate. Products marked with * are factor IX concentrates. All others are factor VIII concentrates.

Correspondence with Alpha Therapeutic Corporation, the manufacturers of coagulation factor concentrates, including the factor VIII concentrates Profilate SD and Alphanate, prompted the addition of 1 IU/ml heparin to the tissue culture medium. Alpha Therapeutic research and development laboratories observed a clot forming while carrying out lymphocyte proliferation assays with their intermediate purity factor VIII concentrate Profilate SD. Their laboratories reported that the addition of 1 IU/ml heparin eliminated this problem and suggested that clot formation interfered with lymphocyte proliferation, producing an artifactual result.

The results demonstrate that PHA-stimulated proliferation of lymphocytes is inhibited by both intermediate purity Profilate SD and affinity purified Alphanate and the addition of heparin does not abrogate inhibition, Figure 25. The only two concentrates which formed clots during incubation were the intermediate purity factor VIII concentrates Z8 and 8Y. This problem was resolved by defibrination of these concentrates prior to inclusion in assays.

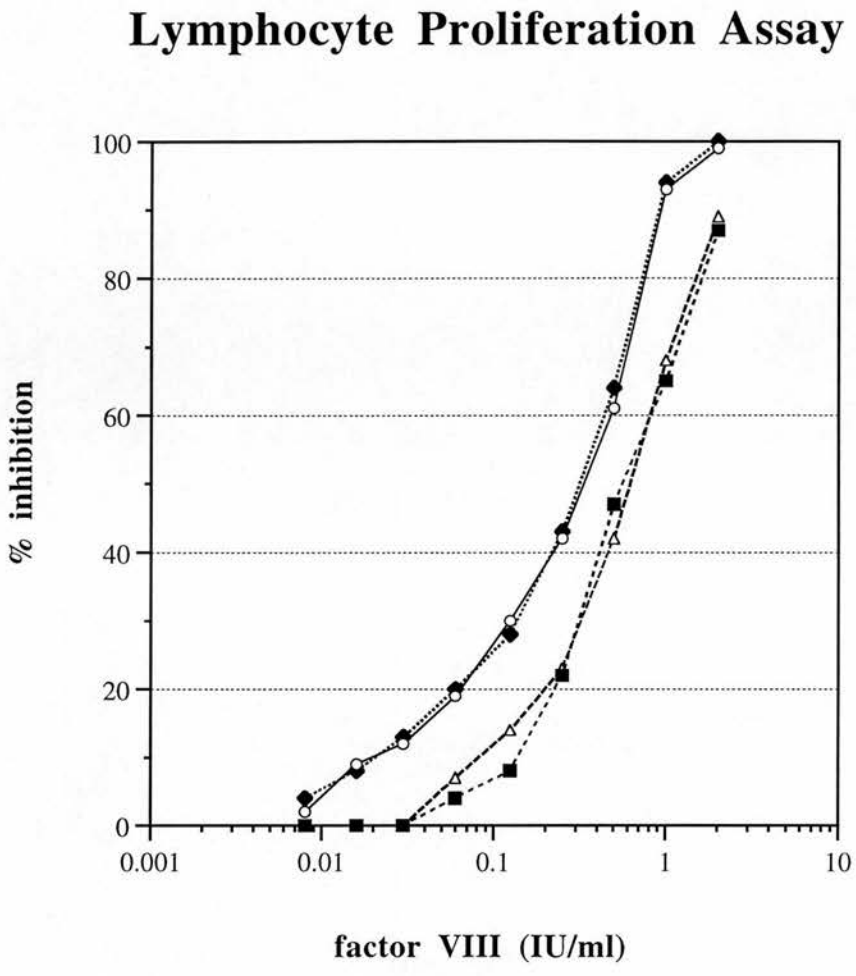


FIGURE 25: Absence of any effect of 1 IU/ml heparin on the inhibition of proliferation of PHA-stimulated lymphocytes by two factor VIII concentrates, intermediate purity Profilate SD (batch AR2003A) and affinity purified Alphanate (AS4701A).

2. Effects of Coagulation Factor Concentrates on IL-2 Secretion by PHA-Stimulated Lymphocytes

The sample of coagulation factor concentrates previously tested in lymphocyte proliferation assays were also tested for their effects on IL-2 secretion by PHA-stimulated lymphocytes, using the method of Thorpe *et al* (1989). Lymphocytes were optimally stimulated with 90 μg/ml PHA in the presence and absence of 1 IU/ml coagulation factor concentrate. Supernatants from these cultures were assayed in triplicate for IL-2 using a bioassay, which is based on the cell line CTLL's dependence on IL-2.

Results were obtained expressed as cpm. The average cpm of each triplicate was calculated and converted to percentage inhibition of IL-2 secretion using the formula on page 73. Where possible, more than one batch of each concentrate was tested.

The results demonstrate that lymphocytes stimulated with PHA produced lower levels of IL-2 in the presence of some coagulation factor concentrates. The percentage inhibition of IL-2 secretion caused by 1 IU/ml of each concentrate is summarised in Table 5. All intermediate purity coagulation factor concentrates inhibited IL-2 secretion when present at a final concentration of 1 IU/ml. The most inhibitory concentrates of this class were the factor IX concentrates Defix and 9A.

Ion-exchange purified coagulation factor concentrates in general did not cause great inhibition of IL-2 secretion, range 0 to 24% inhibition of IL-2 secretion, Table 5.

As with the lymphocyte proliferation assay results, the effects on IL-2 secretion by the two affinity purified products differed considerably, Alphanate causing greater inhibition than 9MC.

The immunoaffinity purified factor VIII concentrates tested in this system caused no inhibition of IL-2 secretion. Recombinant products were not tested in this system.

The percentage inhibition of lymphocyte proliferation for each concentrate was found to correlate with the percentage inhibition of IL-2 secretion. Using the Spearman rank test the correlation of the two variables was 0.703 ($p < 0.01$).

Purification	Product	Batch Number	% Inhibition of Interleukin 2 Secretion
Intermediate	Profilate SD	AR2003A	31
	Profilate SD	AR2008A	26
	Profilate OSD	AR4205A	56
	Haemate P	978641	89
	Defix*	20720	98
	9A*	FJA 0114	95
Range			**26 - 98**
Ion-exchange	HPVIII	20390	13
	HPVIII	30560	0
	HPVIII	30650	24
	HPVIII	40820	21
	Immunate	09H309210S	0
	Immunate	09H319210S	4
	Immunate	09H319301S	19
	Immunate	09H349211S	2
	Immunate	09H359212S	11
	AlphaNine SD*	CA21401A	11
	AlphaNine SD*	CA2403AB	20
Range			**0 - 24**
Affinity	Alphanate	AS4701A	25
	9 MC*	PMC 2203	7
Immunoaffinity	8 SM	FHD 4143	0
	Monoclate P	J53706	0

TABLE 5: Interleukin 2 bioassay results.

Percentage inhibition of interleukin 2 secretion, represents values obtained at 1 IU/ml of clotting factor concentrate. Products marked with * are factor IX concentrates. All others are factor VIII concentrates.

DISCUSSION

1. Effects of Coagulation Factor Concentrates on PHA-Stimulated Lymphocyte Proliferation

As previously stated lymphocyte proliferation in response to the mitogen PHA has been used as an assay to measure the effects that intermediate purity coagulation factor concentrates have on immune function. However, limited data was available regarding the effects of concentrates prepared by chromatography, immunoaffinity or recombinant techniques. Therefore it was seen to be necessary to test all the current purities of product available in this system.

Since, the experimental conditions for PHA-stimulated lymphocyte proliferation assays used by other researchers tend to vary, preliminary experiments to determine optimal assay conditions were carried out. Optimisation experiments clearly demonstrate that experimental conditions such as PHA concentration can significantly affect the outcome, Figure 5. With increasing PHA concentration a reduction in inhibition of proliferation by the coagulation factor is observed. This suggests that the degree to which coagulation factor concentrates inhibit lymphocyte proliferation is somewhat determined by PHA concentration. Nonetheless, the concentrates continue to inhibit PHA-stimulated lymphocyte proliferation to some extent until the PHA concentration becomes toxic, implying that the inhibitory effect of concentrates is not totally dependent on PHA concentration. These observation suggest that data obtained using different expreimental conditions cannot be relaibly compared.

Following optimisation of the assay, the coagulation factor concentrates selected for study were tested over a range of concentrations which are readily achievable during replacement therapy. In agreement with previous studies (Frobel *et al*, 1983; McDonald *et al*, 1985; Lederman *et al*, 1986), intermediate purity factor VIII and factor IX concentrates were found to inhibit PHA-stimulated lymphocyte proliferation in a dose dependent manner, Figures 7-12. In addition, I have demonstrated that some ion-exchange chromatography and affinity purified concentrates also cause inhibition of lymphocyte proliferation; whereas, those

purified by immunoaffinity and recombinant techniques do not, Figures 13-24. All factor VIII, factor IX and von Willebrand factor concentrates of intermediate purity and some of those purified by ion-exchange chromatography inhibited lymphocyte proliferation. Concentrates had no observed effect on non-stimulated lymphocytes.

A general trend was observed whereby, as the product purity increased the inhibition of proliferation decreased, Tables 4(a)-4(c) Thus, *in vitro* the purer the product the less immune modulating activity it possess. A few exceptions to this generalisation were noted. For example one batch of the intermediate purity factor VIII concentrate 8Y, possesses notably less activity than the other two batches tested. Differences in inhibitory activities between products belonging to the same purification group are perhaps not surprising, since variations in manufacturing techniques will inevitably lead to differences in the abundance or molecular weight of contaminants, this being reflected in the values of percentage inhibition of lymphocyte proliferation.

2. Mechanism of Inhibition of PHA-Stimulated Lymphocyte Proliferation by Coagulation Factor Concentrates

Having established that some coagulation factor concentrates have the ability to inhibit lymphocyte mitogenic responses, the mechanism of inhibition was investigated. Cytotoxicty of intermediate and ion-exchange purity factor VIII concentrates on PHA-stimulated lymphocytes had been examined by other researchers and concentrates of both levels of purity found not to mediate their inhibitory effects on lymphocyte proliferation by direct cytotoxicity. However, the effects of other coagulation factor concentrates, or of those purified by affinity techniques had not been examined.

In agreement with previous studies, the inhibition of proliferation caused by intermediate and ion-exchange factor VIII concentrates was found not to be caused by direct cytotoxicity, as the viability of PHA-stimulated lymphocytes incubated with the concentrate was the same as in the control culture (data not shown). Other inhibitory coagulation factor concentrates, factor IX and von Willebrand factor

concentrates did not affect cell viability, neither did the inhibitory affinity purified concentrate Alphanate (data not shown).

It has been suggested that the addition of 1 U/ml heparin to lymphocyte proliferation assays when testing coagulation factor concentrates eliminated all inhibitory effects (personal communication from Alpha Therapeutic Corporation). It had been noted, on testing the intermediate purity concentrate, Profilate SD, in similar PHA-stimulated lymphocyte proliferation assays, that a clot formed, which interfered with lymphocyte proliferation, producing an artifactual result. In my experience, no clotting was observed with any coagulation factor concentrate, of any purity, in any assay, and furthermore the addition of heparin to lymphocyte proliferation assays did not reverse the inhibitory effect of Profilate SD nor that of their affinity purified factor concentrate Alphanate. From these results it can be concluded that the inhibition of lymphocyte proliferation caused by coagulation factor concentrates is not mediated either by the coagulation factors themselves nor by cytotoxic effects. This is supported by Lederman *et al* (1986) who found that inhibition of PHA-stimulated lymphocyte proliferation was not mediated by the coagulant effects of preparations. Lederman *et al* reported that concentrations of heparin and plasmin sufficient to completely inhibit factor VIII activity did not abrogate the inhibitory effects of intermediate purity factor VIII concentrates.

3. Effects of Coagulation Factor Concentrates on IL-2 Secretion by PHA-Stimulated Lymphocytes

The same range of concentrates tested in PHA-stimulated lymphocyte proliferation assays was also tested for effects on IL-2 secretion by PHA-stimulated lymphocytes. Only concentrates of intermediate purity were observed to cause significant inhibition of IL-2 secretion by lymphocytes. Those purified by ion-exchange, affinity or immunoaffinity techniques did not cause significant inhibition of IL-2 secretion, Table 5. Unfortunately, recombinant products were not available at the time for testing in this system.

On comparison of these results to published data (Wadhwa *et al*, 1992), it can be seen that where identical concentrates are used (although on different batch numbers) the results are in close agreement. However, the overview of results from the Wadhwa *et al* paper would to suggest that there is no obvious relationship between product purity and inhibition of IL-2 secretion. In contrast, I have observed a general trend whereby, as the specificity of the manufacturing technique increased, (and hence product purity increased) the mean inhibition of IL-2 secretion by PHA-stimulated lymphocytes for each product group decreased. These conflicting conclusions may be due to different concentrates being used to represent each purity group. However, my conclusion is supported by the trend also shown by lymphocyte proliferation assay data, where again the purer the product the less inhibitory effects it displays.

The percentage inhibition of lymphocyte proliferation and percentage inhibition of IL-2 secretion by each coagulation factor concentrate was found to correlate, using the Spearman rank test. This infers that results obtained by either of my assay systems are directly comparable and are measuring a similar inhibitory phenomenon. There are exceptions where the inhibition of proliferation and IL-2 secretion do not correlate. For example, six batches of the ion-exchange purified factor VIII concentrate Immunate were found to cause profound inhibition of lymphocyte proliferation but had no effect on IL-2 secretion. This suggests that in these cases, the assays are perhaps measuring slightly different proportions of the same contaminants or different biological activities resulting from different inhibitory contaminants. These discrepancies may also be due to the fact that when observing lymphocyte proliferation one is taking into consideration all mechanisms affecting cellular proliferation, whereas with IL-2 secretion only one pathway of inhibition is considered.

From this series of experiments, which have assessed the immune modulating capabilities of coagulation factor concentrates of varying purity, it can be concluded that the purer the product the less effect it will have on lymphocyte function *in vitro*. Whether this is the case for lymphocyte function *in vivo*, is the subject of intensive debate.

As this thesis was aimed at investigating the nature of immunomodulatory contaminants in coagulation factor concentrates, an assay system had to be chosen in which to test manipulations of these concentrates for such activities. The two assay systems described thus far both have drawbacks. However, in comparison to the assay which measured inhibition of IL-2 secretion, the lymphocyte proliferation assay is less labour intensive. It does not require the use of cell lines which have their own drawbacks such as infection with mycoplasma and loss of response to the cytokine of interest. In addition, lymphocyte proliferation appears to be more sensitive to the inhibitory effects of concentrates. This is perhaps because as previously suggested, with lymphocyte IL-2 secretion only one mechanism of inhibition is being observed. Whereas, with lymphocyte proliferation all mechanisms leading to reduced lymphocyte transformation are being measured. Taking the above into consideration, the PHA-stimulated lymphocyte proliferation assay was chosen for use in the further study of immunomodulatory activity in factor VIII concentrates.

4. Effects of Coagulation Factor Concentrates on IL-6 Secretion by PHA-Stimulated Lymphocyte Proliferation

As previously discussed, the cytokine IL-6 is also secreted by lymphocytes and its production up-regulated by both mitogenic stimulation and by IL-2. Therefore, it would follow that if IL-2 secretion is inhibited by some coagulation factor concentrate, IL-6 secretion will alo be reduced.

I have examined the effects of factor VIII, IX and von Willebrand factor concentrates of varying purity on IL-6 secretion by PHA-stimulated lymphocytes and measured the bioactivity of IL-6 using a [methyl-^{3}H]thymidine incorporation assay based on the growth response of the IL-6 dependent murine B cell line B9. The results obtained demonstrated that concentrates did not affect IL-6 production as was expected (data not shown). Nevertheless, these results agree with Wadhwa *et al* (unpublished data, Wadhwa *et al*, 1994) who also demonstrated that factor VIII concentrates did not effect IL-6 secretion.

CHAPTER 4

Is TGF-β Involved in Factor VIII Induced Suppression of Immune Function?

INTRODUCTION

In September 1994 a report was published by Wadhwa *et al* which identified TGF-β as a major contaminant in some factor VIII concentrates, and claimed that it is a major contributory factor to the inhibitory effect on cytokine secretion shown by some concentrates. Wadhwa *et al* (1992) had previously reported that some, but not all, intermediate purity factor VIII concentrates and those purified by ion-exchange chromatography inhibit IL-2 secretion from PHA-stimulated T lymphocytes. Using a specific bioassay, TGF-β levels were estimated in the various factor VIII concentrates. These levels were found to strongly correlate with the degree of inhibition of IL-2 secretion from T lymphocytes exhibited by each product. No detectable TGF-β was found in non-inhibitory concentrates. Furthermore, when an antibody specific to TGF-β1 was included in IL-2 bioassays along with the factor VIII concentrates, the inhibitory effects of some concentrates was partially reversed. From the results obtained in TGF-β neutralising experiments, the authors concluded that TGF-β is a major contributory factor to the inhibitory activity of some concentrates on cytokine secretion and activity.

I have measured the levels of biologically active TGF-β and latent TGF-β1 in the same sample of coagulation factor concentrates that had previously been assessed for their ability to inhibit the function of PHA-stimulated lymphocytes. Active TGF-β levels were measured by a bioassay which was based on the ability of TGF-β to inhibit the proliferation of the cell line Mv-1-Lu (Tucker *et al*, 1978; Like & Massagué, 1986). TGF-β is also found circulating in plasma as a latent complex which is biologically inactive and undetectable by bioassay. Therefore, in order to determine if any latent TGF-β was present in coagulation factor concentrates TGF-β1 was measured by ELISA.

Having confirmed that whether active and/or latent TGF-β is present in the sample of coagulation factor concentrates, it remained to be determined whether its presence was responsible for the inhibition of lymphocyte function *in vitro*. In order to ascertain whether TGF-β contamination alone was responsible for inhibiting

PHA-stimulated lymphocyte proliferation, TGF-β neutralisation was performed during lymphocyte proliferation with various concentrates. Here data are presented which clearly demonstrates that TGF-β has little part in the inhibition of PHA-stimulated lymphocyte proliferation caused by factor VIII concentrates.

In this chapter the potential impact that levels of TGF-β found in concentrates would have on an individual receiving coagulation factor therapy was also investigated.

MATERIALS AND METHODS

1. Cells

1.1 Preparation of T Lymphocytes

T lymphocytes were separated from PBMC (prepared as described on page 85) by sheep red blood corpuscle (SRBC)-rosetting (Londei *et al*, 1991). Sheep blood from healthy non-immunised animals collected in modified Alsevers solution to provide a 50% vol/vol mixture (Scottish Antibody Production Unit {S.A.P.U.}, Carluke, UK) was layered over Lymphoprep and centrifuged at 800g for 15 minutes. One ml of the packed red cell pellet was removed and washed three times in sterile saline. The final red cell pellet was resuspended in 15 ml of a 2% solution of S-2-aminoethylisothiouronium bromide (AET) then incubated at 37°C for 15 minutes, shaking every fifth minute. The AET solution was freshly prepared, adjusted to pH 8.0 and sterile filtered (μStar. Pore diameter 0.2 μm. Costar Ltd, UK) before use. The resulting AET treated SRBC were washed four times in saline before being resuspended in 10 ml of RPMI 1640 medium containing 40% FCS to give a final 10% suspension.

PBMC were prepared as described previously and adherent cells removed by adjusting the concentration of the suspension to $1x10^6$ cells/ml in RPMI 1640 medium containing 10% FCS and incubating at 37°C for 1 hour in an 80 cm^2 culture flask (Costar Ltd, UK). The remaining non-adherent cells were then adjusted to a concentration of $4x10^7$ cells/ml in RPMI 1640 medium containing 40% FCS and mixed with an equal volume of the freshly prepared AET-SRBC. Aliquots of 1 ml of suspension were placed in round bottomed tubes (Costar Ltd.), centrifuged at 150g for 5 minutes then stored at 4°C for 60 minutes. The pellets were gently resuspended, layered over Lymphoprep and centrifuged at 800g for 15 minutes. The interface, containing non-rosetted mononuclear cells (B lymphocyte enriched population) was discarded. T lymphocytes were freed from rosetting SRBC by lysis

of the SRBC by adding 10 ml of a 0.17 M ammonium chloride solution, and placing on ice for 10 minutes. The T lymphocytes were washed once in RPMI 1640 medium containing 10% FCS, twice in sterile phosphate buffered saline (PBS), and used immediately.

1.2 Origin and Maintenance of Mv-1-Lu Cells

The mink lung epithelial cell line Mv-1-Lu was obtained from ECACC (ECACC No: 8805) for use in TGF-β bioassay. This cell line was maintained on RPMI 1640 medium with 5% FCS in 80 cm^2 culture flasks. Confluent cell sheets were washed twice in sterile PBS and sufficient 0.25% trypsin-EDTA solution (Sigma-Aldrich Ltd.) added to cover the cells. Flasks were incubated at 37°C until the cell sheet detached. The resulting cell suspension was washed three times in RPMI 1640 containing 5% FCS. Cells were split in a ratio of 1:6 and resuspended in culture medium.

2. Antibodies

Antibodies to TGF-β were obtained from two sources for use in neutralisation experiments. Dr. Meenu Wadhwa (National Institute of Biological Standards and Control) kindly donated the antibody used in her study which identified TGF-β as a contaminant in factor VIII concentrates. According to her data, a 1:200 dilution of the purified chicken, polyclonal anti-TGF-β1 antibody was capable of neutralising 5 ng/ml TGF-β1. A commercial polyclonal neutralising antibody to TGF-β produced by R & D Systems Europe, was also used in neutralising experiments. According to manufacturers' data, 50 μg/ml of this antibody was capable of neutralising 7.5 ng/ml TGF-β1.

3. Proliferation Assays

3.1 PHA-Stimulated Lymphocyte and T Lymphocyte Proliferation Assays

Proliferative responses to 1 μg/ml PHA were measured by culturing PBMC or purified T lymphocytes in 96-well flat-bottomed microtitre plates. For this purpose, a cell suspension of $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FCS was prepared. Triplicate wells containing 100 μl of cell suspension and 100 μl of serial doubling dilutions of coagulation factor concentrates (0.004-2 IU/ml) or other test samples were prepared in culture medium. Again the range of coagulation factor concentrate activity covered *in vitro* was chosen to reflect plasma levels in patients following replacement therapy. Control cultures of cells in culture medium alone, cells and PHA alone, and cells plus coagulation factor concentrate in the absence of PHA, were included in each assay. Cultures were incubated for 96 hours before harvesting. Proliferation of cells in response to PHA was measured by pulsing cultures with 1μCi/well of [methyl-^{3}H]thymidine 18 hours before cell harvesting.

3.2 Neutralising Lymphocyte Proliferation Assays

Contaminating TGF-β in coagulation factor concentrates was neutralised using three different approaches.

Method 1:- Triplicate wells containing a total volume of 100 μl which contained a 1:200 dilution of purified chicken anti-TGF-β1 or dilutions of the commercial neutralising antibody to TGF-β and 1 or 2 IU/ml of coagulation factor concentrate were incubated at 37°C for 1 hour before the addition of PBMC. A suspension of PBMC containing $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FCS was prepared and 100 μl added to each well.

Method 2:- Serial doubling dilutions of pan-specific TGF-β neutralising antibody (R & D Systems Europe Ltd) or control IgG (S.A.P.U.) in 100 µl volumes were prepared in triplicate, in 96-well flat-bottomed plates. To each antibody dilution 1 ng/ml of either TGF-β1 in purified form (R & D Systems Europe Ltd.) or TGF-β contained in coagulation factor concentrate was added. Plates were incubated at 37°C for 1 hour before addition of PBMC. A PBMC suspension containing $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FCS was prepared and 100 µl added to each well.

Method 3:- Serial doubling dilutions of a the commercial polyclonal TGF-β neutralising antibody or control IgG (S.A.P.U.) in 100 µl volumes were prepared in triplicate, in 96-well flat-bottomed plates. An equivalent inhibitory amount of purified TGF-β1 or concentrate containing TGF-β was added to dilutions of the commercial neutralising antibody to TGF-β. The amount of coagulation factor and TGF-β1 to be added was assessed by T lymphocyte proliferation assay. Plates were incubated at 37°C for 1 hour before addition of the T lymphocytes. A T lymphocyte suspension containing $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FCS was prepared and 100 µl added to each well.

Control cultures of cells in culture medium alone, cells and PHA alone, and PHA stimulated cells plus coagulation factor concentrate in the absence of antibody, were included in every experiment. Proliferation was stimulated with PHA which was included at a final concentration of 1 µg/ml. All cultures were incubated for 96 hours before harvesting. Proliferation was measured by pulsing each well with 1 µCi of [methyl-^{3}H]thymidine 18 hours before cell harvesting.

4. Cytokine Bioassays

4.1 Bioassay for Transforming Growth Factor Beta

TGF-β was measured using a bioassay based on the ability of TGF-β to inhibit the proliferation of the epithelial cell line Mv-1-Lu. For this purpose, Mv-1-Lu cells were trypsinised as described previously, washed three times in RPMI 1640 and resuspended at $2x10^4$ cells/ml in RPMI 1640 medium containing 5% FCS. Triplicate wells in 96-well flat-bottomed plates were prepared containing 100 µl of cell suspension and 100 µl serial doubling dilutions of coagulation factor concentrate (0.004-2 IU/ml), or test sample in culture medium. A titration of a standard purified human TGF-β1 was included in every assay. Cultures were incubated for 4 days before pulsing with 1 µCi/well [methyl-^{3}H]thymidine 5 hours prior to cell harvesting. In order to remove the cells before harvesting, wells were washed twice with 200 µl PBS and 200 µl of 0.25% trypsin-EDTA solution added 15 minutes before harvesting. A dose response curve of percentage inhibition versus the log of dilution of purified human TGF-β1 standard or coagulation factor concentrate was plotted and the amount of active TGF-β1 estimated by comparison of the coagulation factor dilution series to the standard curve, using parallel line analysis (European Pharmacopoeia, 1971).

4.2 Neutralising Bioassay for TGF-β

For this purpose, serial doubling dilutions of the commercial polyclonal neutralising to TGF-β or control IgG in 100 µl volumes were prepared in triplicate, in 96-well flat-bottomed plates. To each dilution equivalent inhibitory amounts of coagulation factor concentrate or purified human TGF-β1 were added, as previously assessed by TGF-β bioassay. Dilutions were incubated at 37°C for 1 hour before the addition of the Mv-1-Lu cells. A suspension of Mv-1-Lu cells containing $2x10^4$ cells/ml in RPMI 1640 medium supplemented with 5% FCS was prepared and 100 µl added to each well. Cultures were incubated for 4 days, then pulsed with 1 µCi/well [methyl-^{3}H]thymidine, for 5 hours prior to harvesting.

5. TGF-β1 Enzyme Linked Immunosorbent Assay

Total TGF-β1 present in serum, plasma and coagulation factor preparations, was measured using the Predicta TGF-β1 kit (Genzyme Diagnostics, UK). This kit is an enzyme linked immunosorbent assay (ELISA) for the quantitative determination of total TGF-β1 in ng/ml. Testing was carried out according to manufacturers' instructions.

A volume of 5 ml of blood from six normal healthy volunteers was collected by venepuncture using a wide bore needle, to minimalise platelet disruption. Plasma was prepared by decanting a 3 ml aliquot into an EDTA tube and centrifuging at 1000g for 10 minutes. Serum was prepared by allowing the remaining 2 ml of blood to clot for 1 hour at 37°C, and centrifuging at 800g for 10 minutes.

A measured volume of acid activated sample, standard, or control was added to each test well and incubated to allow any TGF-β1 present to be bound by the mouse monoclonal antibody to TGF-β1, immobilised on the microtiter plate. The wells were washed and a direct-labelled horse radish peroxidase (HRP)-conjugated polyclonal antibody to TGF-β1 added which bound to the captured TGF-β1 during further incubation. After washing, a substrate solution was added to the wells, which produced a blue colour in the presence of peroxidase. The colour reaction was stopped by the addition of acid which changed the blue colour to yellow. The intensity of the yellow colour is directly proportional to the amount of TGF-β1 present in the sample. The absorbance of each well was read at 450 nm in a Titertek Multiscan ELISA plate reader (Life Sciences Inrenational Ltd, UK), and a standard curve constructed to quantitate TGF-β1 concentration in the controls and samples.

RESULTS

1. Estimation of Active TGF-β Levels in Coagulation Factor Concentrates

The coagulation factor concentrates which had previously been tested for their ability to inhibit lymphocyte function were also tested in a TGF-β bioassay in order to determine whether biologically active TGF-β was a contaminant. A bioassay based on the ability of TGF-β to inhibit the growth of Mv-1-Lu, mink lung epithelial cells, was used. As mentioned previously, the TGF-β family in man consists of TGF-β1, TGF-β2 and TGF-β3. These different TGF-β subtypes all inhibit the proliferation of Mv-1-Lu cells, albeit variably (Arrick *et al*, 1990). Therefore, it must be assumed that this bioassay is measuring active TGF-β1, TGF-β2 and TGF-β3.

Coagulation factor concentrates of varying purity were tested in triplicate over a range of dilutions (0.004-2 IU/ml). Where possible, more than one batch of each concentrate was tested. Results obtained were expressed as cpm and percentage inhibition calculated using the equation shown on page 78. Following conversion, the data were then plotted graphically as for lymphocyte proliferation assays (page 95). A representative graph, demonstrating the inhibitory effects that two batches of an intermediate purity factor VIII concentrate have on Mv-1-Lu cell proliferation, is shown in Figure 26. The inhibition of Mv-1-Lu proliferation by coagulation factor concentrates was found to be dose dependent.

For each experiment a standard curve was constructed of log purified human TGF-β (0.001-5 ng/ml) versus percentage inhibition of proliferation. This curve was used to estimate levels of active TGF-β in concentrates by parallel line analysis. A typical standard curve of inhibition of Mv-1-Lu proliferation by TGF-β1 is shown in Figure 27.

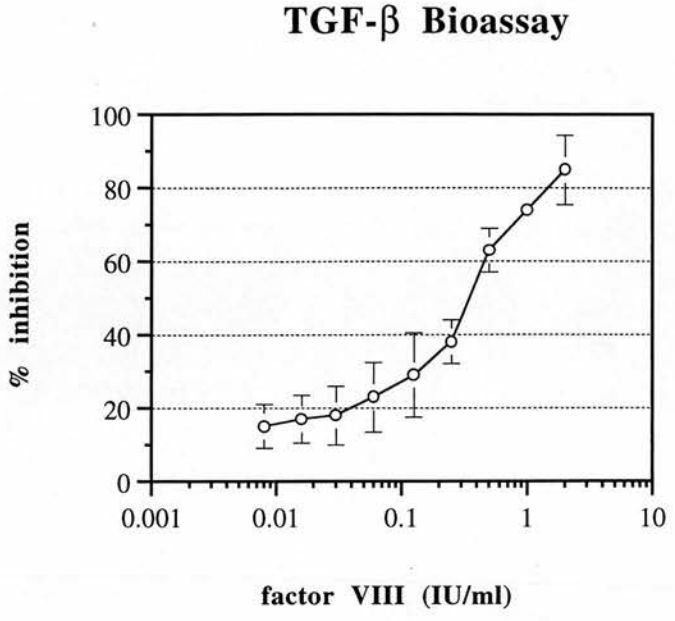


FIGURE 26: Representative graph showing the inhibition of Mv-1-Lu cell proliferation by two batches of an intermediate purity factor VIII concentrate, 8Y.

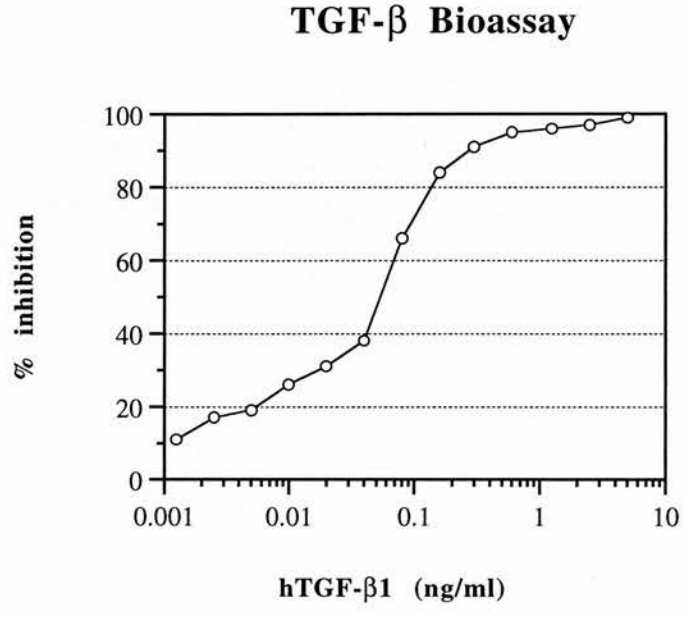


FIGURE 27: Inhibition of Mv-1-Lu cell proliferation by a standarised preparation of purified human TGF-β1.

The levels of active TGF-β measured in each concentrate are shown in Tables 7(a), 7(b) and 7(c). All intermediate purity coagulation factor concentrates inhibited Mv-1-Lu proliferation and hence contained biologically active TGF-β, Table 7(a). Both factor VIII and factor IX concentrates manufactured by conventional methods contained active TGF-β.

The effects of ion-exchange purified products on Mv-1-Lu cell proliferation, and the amounts of active TGF-β contained in these products varied greatly, Table 7(b). The factor VIII concentrate, HPVIII and the vWF concentrate Concentré De Facteur Willebrand both contained active TGF-β. However, the factor VIII concentrate Immunate also prepared by ion-exchange, did not inhibit Mv-1-Lu proliferation and hence did not contain any active TGF-β. Unlike other concentrates, the factor IX concentrate AlphaNine SD enhanced Mv-1-Lu cell proliferation. When present at a final concentration of 2 IU/ml, both batches of this concentrate caused a 100% increase in Mv-1-Lu cell proliferation in comparison with control cultures, Figures 28.

This enhancement of Mv-1-Lu proliferation was also observed with three batches of the affinity purified factor VIII concentrate Alpha VIII, Figure 29. This effect appears to be a specific to some concentrates manufactured by Alpha Therapeutic, since, it was not seen with any other product. The amounts of active TGF-β that the other affinity purified products contained was variable. No active TGF-β was detected in the affinity purified product 9MC. However, a high level of active TGF-β (4.61 ng/ml) was detected the factor VIII product Alphanate, Table 7(c).

Products produced by immunoaffinity and those manufactured by recombinant methods, contained no detectable biologically active TGF-β.

The amount of active TGF-β present in coagulation factor concentrates was found to correlate with both percentage inhibition of proliferation and percentage inhibition of IL-2 secretion by PHA-stimulated lymphocytes. Using the Spearman rank test the correlation of active TGF-β and percentage inhibition of lymphocyte proliferation was 0.546 (p=0.01), while that for active TGF-β and percentage inhibition of IL-2 secretion was 0.768 (p=0.001).

Purification	Product	Batch Number	Active TGF-β (ng/ml)	Total TGF-β1 (ng/ml)
Intermediate	Z8	1-378	0.58	3.47
	Profilate SD	AR2003A	1.98	10.0
	Profilate SD	AR2004A	3.61	9.77
	Profilate SD	AR2008A	ND	10.7
	Profilate OSD	AR4205A	4.61	5.00
	8Y	FHC 0494	4.05	0.43
	8Y	FHC 4133	3.84	0.43
	Haemate P	978641	3.40	0.65
	Defix*	20720	1.87	7.60
	9A*	FJA 0114	3.85	5.00
Range			**1.58 - 4.61**	**0.43 - 10.7**

TABLE 7(a): TGF-β bioassay and TGF-β1 ELISA results for intermediate purity products.

Active TGF-β levels were assessed by TGF-β bioassay. Total TGF-β1 values were assessed by TGF-β1 ELISA. Products marked with * are factor IX concentrates, all others are factor VIII concentrates. Abbreviation: ND = not done.

Purification	Product	Batch Number	Active TGF-β (ng/ml)	Total TGF-β1 (ng/ml)
Ion-exchange	HPVIII	10040	1.22	0.54
	HPVIII	20390	3.29	3.36
	HPVIII	30540	2.53	2.17
	HPVIII	30560	1.42	4.32
	HPVIII	30650	1.44	3.24
	HPVIII	40820	1.97	3.10
	Immunate	09H309210S	0	1.36
	Immunate	09H319212S	0	1.57
	Immunate	09H319301S	0	1.09
	Immunate	09H349211S	0	0.22
	Immunate	09H359212S	0	1.63
	AlphaNine SD*	CA21401A	NA	0.16
	AlphaNine SD*	CA2403AB	NA	0.11
	Concentré De Facteur Willebrand#	87920070	1.36	0.65
Range			**0 - 3.29**	**0.11 - 4.32**

TABLE 7(b): TGF-β bioassay and TGF-β1 ELISA results for ion-exchange products.

Active TGF-β levels were assessed by TGF-β bioassay. Total TGF-β1 values were assessed by TGF-β1 ELISA. Products marked with * are factor IX concentrates, those marked with # are von Willebrand concentrates. All others are factor VIII concentrates. Abbreviations: ND = not done, NA = not applicable.

Purification	Product	Batch Number	Active TGF-β (ng/ml)	Total TGF-β1 (ng/ml)
Affinity	Alpha VIII	AP2005A	NA	8.25
	Alpha VIII	AP2006A	NA	ND
	Alpha VIII	AP2008A	NA	9.77
	Alphanate	AS4701A	4.61	5.94
	9 MC*	PMC 2203	0	0.11
Immunoaffinity	8 SM	FHD 4143	0	0.22
	Monoclate P	J53706	0	0.49
Recombinant	Kogenate	ADAH1	0	ND
	Recombinate	95F22A250	0	ND

TABLE 7(c): Summary of TGF-β bioassay and TGF-β1 ELISA results for affinity purified, immunoaffinity purified and recombinant products.

Active TGF-β values were assessed by TGF-β bioassay. Total TGF-β1 values were assessed by TGF-β1 ELISA. Abbreviations: ND = not done.

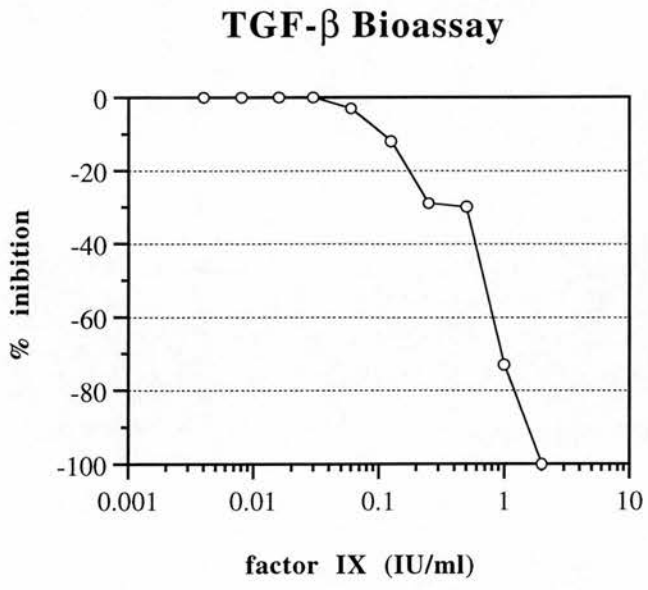


FIGURE 28: Enhancement of Mv-1-Lu cell proliferation (expressed as negative inhibition) by two batches of the ion-exchange purified factor IX product AlphaNine SD. (Error bars too close to points to be illustrated.)

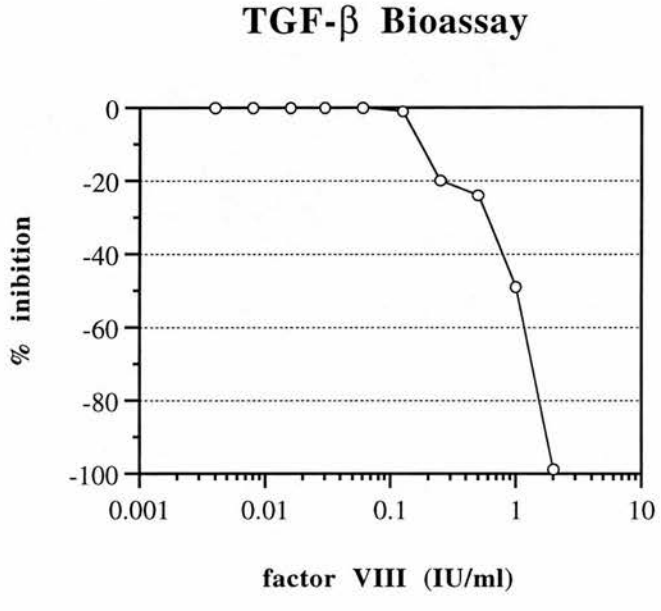


FIGURE 29: Enhancement of Mv-1-Lu cell proliferation (expressed as negative inhibition) by three batches of the affinity purified factor VIII Alpha VIII. (Error bars too close to points to be illustrated.)

A common drawback of bioassays is that the cell lines used are not absolutely specific for the cytokine of interest. For example, the growth of Mv-1-Lu cells is also variably inhibited by TNF α and IFN α, as shown in Figure 30. Besides other cytokines, serum proteins such as α_2M are known to affect TGF-β mediated growth inhibition. In the mink lung bioassay α_2M has been shown to inhibit the activity of TGF-β2, however it has no effect on TGF-β1 (Dannielpour & Sporn, 1990). In addition, TGF-β is abundant in FCS. Thus, it follows that FCS will have a profound effect on Mv-1-Lu cell proliferation. For this reason, different batches of FCS were tested to identify one which allowed optimal inhibition with purified TGF-β1 (data not shown).

In order to ascertain the specificity of this assay, dilutions of some of the concentrates tested were incubated with a neutralising polyclonal antibody specific to TGF-β. The contaminating TGF-β present in two factor VIII concentrates, HPVIII (ion-exchange purified) and Profilate OSD (conventional fractionation) was neutralised, using a commercial neutralising polyclonal antibody to TGF-β. Preliminary bioassays were carried out to identify concentrations of HPVIII (IU/ml) and purified human TGF-β1 (ng/ml) which were equivalent inhibitors of Mv-1-Lu proliferation. For example, Figures 31 and 32 demonstrate that 2 IU/ml of HPVIII and 0.16 ng/ml TGF-β1 cause equivalent inhibition of Mv-1-Lu cell proliferation. These equivalent inhibitory concentrations of concentrate and purified TGF-β1 were incubated with increasing amounts of antibody to TGF-β, prior to the addition of Mv-1-Lu cells. The highest concentration of antibody (100 μg/ml) reduced the inhibition of HPVIII by 80% and TGF-β by 79%, effectively neutralising the inhibitory activity of both samples, Figure 33. These results suggest that all the Mv-1-Lu proliferation inhibitory activity of HPVIII is due to the presence of TGF-β, and not to some other cytokine.

The neutralisation of control purified TGF-β1 by the antibody to TGF-β verifies that the antibody is indeed neutralising the activity of TGF-β. When cultured with Mv-1-Lu cells in the absence of concentrate, both the antibody to TGF-β and the control antibody, at the concentrations used, had no effect on proliferation (data not

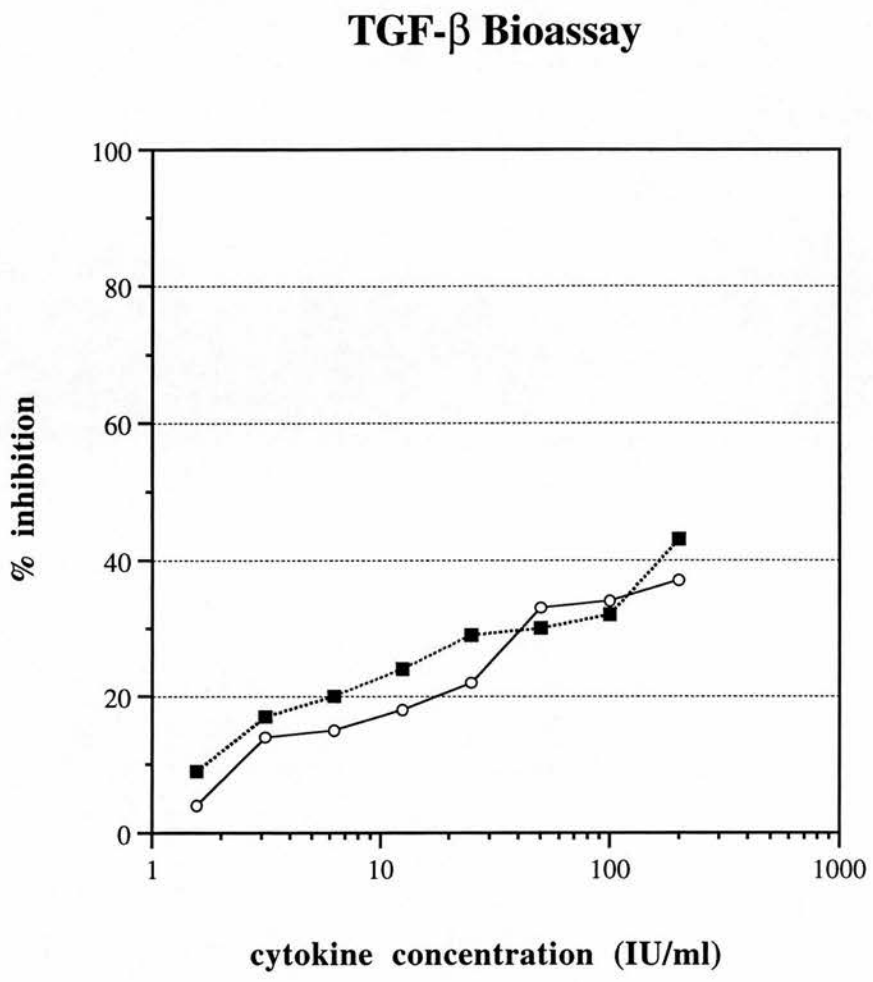


FIGURE 30: Inhibition of Mv-1-Lu cell proliferation by TNF α and IFN α.

—o— TNF α

·····■····· IFN α

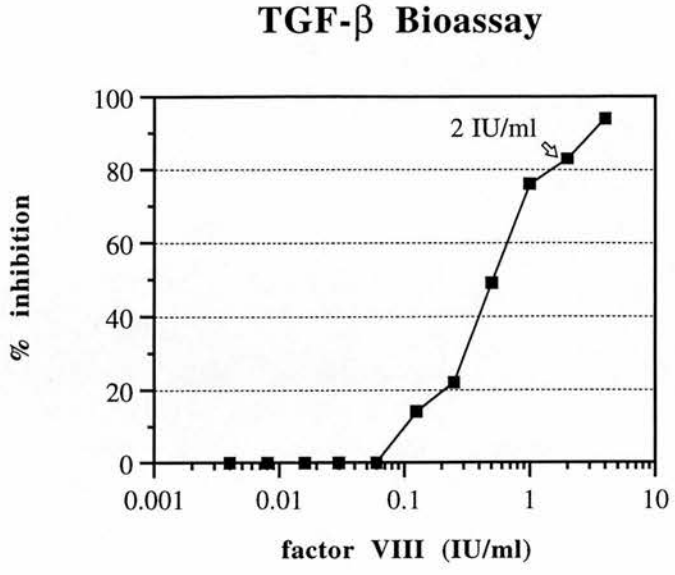


FIGURE 31: Inhibition of Mv-1-Lu cell proliferation by HPVIII (batch 30540).

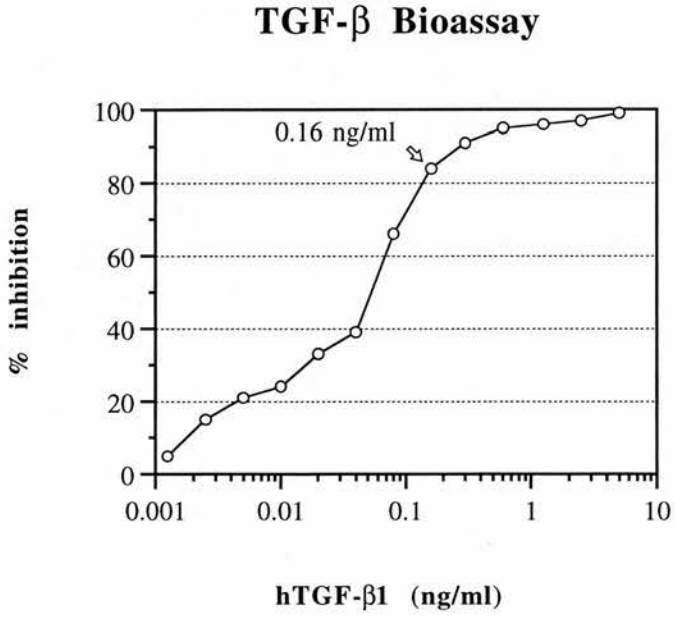


FIGURE 32: Inhibition of Mv-1-Lu cell proliferation by purified human TGF-β1. From these experiments it can be seen that HPVIII (batch 30540, 2 IU/ml) and human TGF-β1 (0.16 ng/ml) are equivalent inhibitors of Mv-1-Lu cell prolferation.

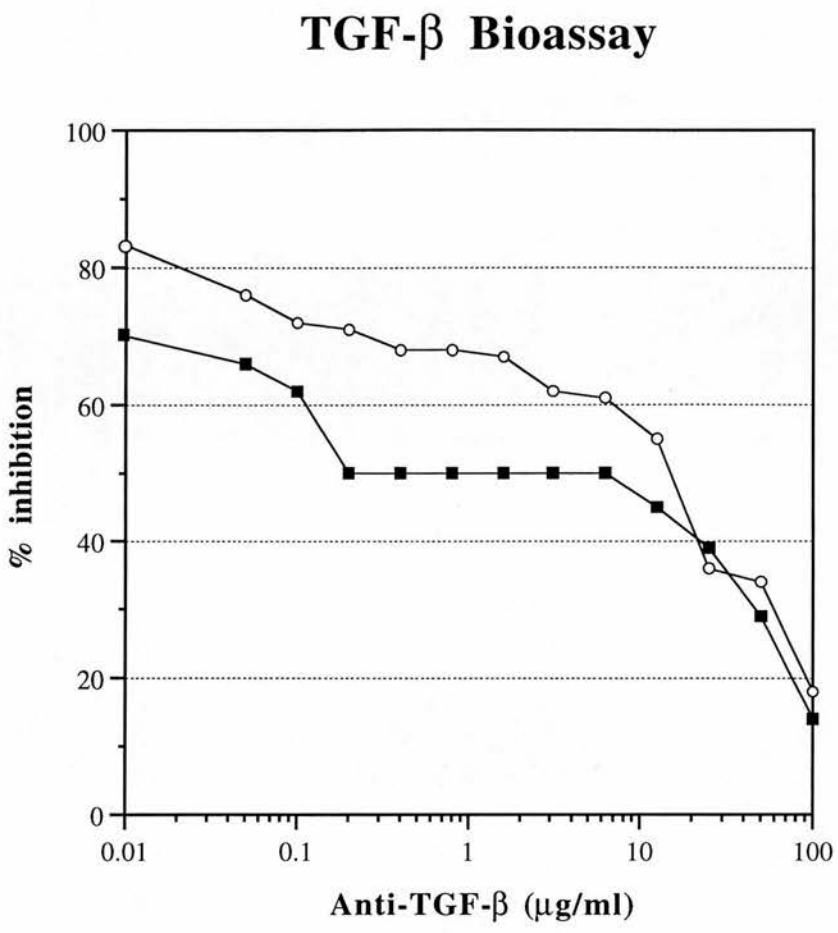


FIGURE 33: Neutralisation of equvalent inhibitory effects of purified human TGF-β1 (0.16 ng/ml) and HPVIII (batch 30540, 2 IU/ml) on Mv-1-Lu cell proliferation by an antibody to TGF-β1.

—■— HPVIII

—○— purified human TGF-β1

shown). Concentrations greater than 100 μg/ml of antibody to TGF-β were found to be toxic to cells.

The same strategy was used to neutralise the TGF-β present in Profilate OSD. Equivalent inhibitory amounts of concentrate and TGF-β were assessed by preliminary bioassay (data not shown) and then neutralised by increasing concentrations of antibody. The highest concentration of antibody (100 μg/ml) reduced the inhibitory effect of Profilate OSD by 69% and that of TGF-β1 by 60% (data not shown), suggesting that the majority of the inhibition of Mv-1-Lu cell proliferation by this concentrate is due to the presence of contaminating TGF-β. The addition of higher concentrations of neutralising antibody to TGF-β may have abrogated a greater portion of the inhibitory activity however, since higher concentrations of the antibody were toxic to Mv-1-Lu cells, this could not be confirmed.

2. Estimation of Total TGF-β1 Levels in Coagulation Factor Concentrates

A commercial TGF-β1 ELISA kit was used to estimate the total TGF-β1 (active and latent) present in the same range of coagulation factor concentrates in which active TGF-β levels had been measured by bioassay.

The results are summarised in Tables 7(a), (b) and (c). TGF-β1 was detected in all intermediate purity coagulation factor concentrates, Table 7(a). In most cases the total TGF-β1 measured was greater than the active TGF-β levels, suggesting the presence of latent, biologically inactive TGF-β1. However, in three cases the total TGF-β1 measured was found to be less than the active TGF-β.

All ion-exchange purified products tested contained some measurable TGF-β1, Table 7(b). The factor VIII product Immunate, which contained no active TGF-β as assessed by bioassay, did however contain latent TGF-β1. With few exceptions, the total TGF-β1 levels found in ion-exchange purified concentrates were greater than active TGF-β levels.

The affinity purified products Alpha VIII and Alphanate contained relatively high amounts of TGF-β1 as assessed by ELISA. Some latent TGF-β was detected in 9MC, even though no active TGF-β was detected.

Immunoaffinity purified products contained small amounts of biologically inactive TGF-β1, Table 7(c). Recombinant products were not tested in this system.

A correlation was found between the levels of active TGF-β and total TGF-β1. Taking each observation as an independent result, the correlation coefficient using the Spearman rank test was 0.485 (p=0.05). The levels of total TGF-β1 in coagulation factor concentrates were also found to correlate with both percentage inhibition of proliferation and IL-2 secretion of PHA-stimulated lymphocytes. The correlation coefficient of total TGF-β1 and percentage inhibition of lymphocyte proliferation was 0.665 ($p < 0.001$), and the correlation between total TGF-β1 and percentage inhibition of IL-2 secretion was 0.567 (p=0.02).

3. Total TGF-β1 Levels in Human Serum and Plasma

In order to predict the impact that the levels of contaminating TGF-β1 in coagulation factor concentrates would have on an individual receiving replacement therapy, total TGF-β1 levels were measured in human plasma and serum.

In the eight normal controls, the mean total TGF-β1 in plasma was 41 ng/ml (range 32-48 ng/ml), and in serum 50 ng/ml (range 21-75 ng/ml).

Assuming the average plasma volume is 40 ml/kg body weight and the average body weight 70 kg, an average individual would have a plasma volume of 2800 ml.

If a haemophiliac of average body weight were to receive 3000 IU/ml of an intermediate purity factor VIII concentrate (20 IU/ml, 150 ml transfused) to treat a bleed, and the concentrate contained 10 ng/ml total TGF-β1, that patient would receive approximately 1500 ng of TGF-β1. If the average plasma level of TGF-β1 is 41 ng/ml as the results suggest, transfusion of this amount would lead to an overall increase of 0.5 ng/ml in the total TGF-β1 levels in plasma, i.e. a change of only 1.3%.

4. Neutralisation of TGF-β in Coagulation Factor Concentrates

To establish whether TGF-β contamination of coagulation factor concentrates was responsible for inhibition of lymphocyte proliferation, TGF-β activity was neutralised by a specific antibody to TGF-β. TGF-β activity in coagulation factor concentrates was neutralised by three different approaches.

The authors of the paper which identified TGF-β as a contaminant in coagulation factor concentrates, Wadhwa *et al*, kindly donated the neutralising antibody used in their study for this purpose. Following the method of Wadhwa *et al*, a concentration of 1 IU/ml of each coagulation factor concentrate was incubated with PHA stimulated lymphocytes in the presence or absence of a 1:200 dilution of the antibody. When present at this concentration, the antibody was capable of neutralising 5 ng/ml of TGF-β1 (Wadhwa *et al*, 1994). Addition of the anti-TGF-β antibody failed to reverse the inhibitory effects that some concentrates have on lymphocyte proliferation, Figure 34. The antibody itself had no effect on PHA-stimulated lymphocyte proliferation (data not shown).

As confirmation that the donated antibody was indeed neutralising TGF-β, a similar experiment was carried out using a commercial polyclonal neutralising antibody to TGF-β. Increasing concentrations of the antibody (0.8 - 100 μg/ml) were incubated with 1 or 2 IU/ml of coagulation factor concentrate prior to the addition of lymphocytes. This batch of antibody according to manufacturers' data, was capable of neutralising 7.5 ng/ml of TGF-β1 when present at 50 μg/ml. As with the donated antibody, the commercial antibody failed to abrogate the effects of three inhibitory concentrates of varying purity, Figure 35. At the concentrations used, the antibody itself had no effect on lymphocyte proliferation (data not shown).

Following these results, a different neutralisation strategy was used, whereby equivalent amounts of purified TGF-β1 and TGF-β-containing factor VIII concentrates (1 ng/ml final concentration of TGF-β) were incubated with increasing concentrations of the commercial neutralising antibody to TGF-β (0.8 - 100 μg/ml), prior to the addition of PHA-stimulated lymphocytes. The antibody, when present even at the high concentration of 100 μg/ml, failed to neutralise any of the inhibitory activity caused by the factor VIII concentrates (data not shown).

It was noted that purified TGF-β1, when present at concentrations of up to 100ng/ml, failed to cause inhibition of PHA-stimulated proliferation (data not shown). Since FCS contains high levels of TGF-β, it may be that a small increase in TGF-β concentration would have no effect on lymphocyte proliferation. For this reason, six different batches of FCS were used to support the growth of PHA-stimulated lymphocytes and the effects of purified TGF-β on proliferation assessed. Purified TGF-β1 was found to have slight inhibitory effects on proliferation in the presence of two of the batches tested (data not shown). Therefore, one of these batches was selected for further experiments. The final percentage of FCS used for proliferation assays was also examined. It was found that a reduction in the percentage of FCS from 10% to 2% brought about a further small increase in the response of PHA-stimulated lymphocyte to purified TGF-β1. However, the counts per minute upon reduction of FCS to 2% were very low (<2000 cpm) and hence unreliable (data not shown). Following these results, the serum free growth supplement insulin-transferrin-sodium selenite (ITSS) was substituted for FCS to eliminate any of the problems possibly associated with levels of cytokines in FCS. However, ITSS failed to provide adequate support for the growth of PHA-stimulated lymphocytes (data not shown).

Several studies on the effects of TGF-β report inhibition of proliferation of T lymphocytes by purified TGF-β1 (Fox *et al*, 1992; Ahuja *et al*, 1993). Therefore, lymphocytes were substituted for purified T lymphocytes in PHA-stimulated proliferation assays. Purified TGF-β1 and an inhibitory intermediate purity factor VIII concentrate, Profilate OSD, were tested in a T lymphocyte proliferation assay. T lymphocyte proliferation was inhibited by both Profilate OSD and TGF-β1, Figure 36. Profilate OSD was known to contain approximately 5 ng/ml TGF-β1, as assessed by both bioassay and ELISA, and, by calculation, the addition of 1 IU/ml of concentrate was equal to the addition of 0.1 ng/ml of TGF-β1. However, addition of equal amounts of purified TGF-β1 and concentrate containing TGF-β1 did not produce the same inhibitory effects, since purified TGF-β1, when present at 0.1 ng/ml, caused 5% inhibition of proliferation, whereas Profilate OSD, when

present at 1 IU/ml (0.1 ng/ml TGF-β1), only caused 96% inhibition of proliferation, Figure 36.

Equivalent inhibitory amounts of this concentrate and purified TGF-β1 were added to increasing concentrations of the commercial TGF-β neutralising antibody and tested in a T lymphocyte proliferation assay. From Figure 36 it can be seen that 0.125 IU/ml of Profilate OSD and 12.5 ng/ml purified TGF-β1 are equivalent inhibitors of PHA stimulated T lymphocyte proliferation. These concentrations of concentrate and TGF-β1 were incubated with the antibody dilutions prior to the addition of T lymphocytes. A semilogarithmic graph of antibody concentration (0.8 - 100 μg/ml) versus percentage inhibition of T lymphocyte proliferation was constructed, Figure 37. The antibody, when present at 50 or 100 μg/ml, neutralisied all the T lymphocyte inhibitory activity of TGF-β1. However, it failed to abrogate all the inhibitory activity caused by the equivalent inhibitory amount of Profilate OSD, reducing it by only 44%.

Purified human TGF-β1 and the inhibitory intermediate purity factor VIII concentrate Profilate SD (batch AR2004A) were also tested in a PHA-stimulated T lymphocyte proliferation assay. T lymphocyte proliferation was inhibited by both the concentrate and the purified TGF-β1, Figure 38. This batch of Profilate SD was known to contain 3.61 ng/ml TGF-β as assessed by bioassay and by calculation, 1 IU/ml of concentrate was equal to the addition of 0.1 ng/ml of TGF-β. Addition of equal amounts of TGF-β1 in purified form or contained in the factor VIII concentrate, to T lymphocytes had markedly different effects. The purified TGF-β1 did not inhibit T lymphocyte proliferation in response to PHA. However, the TGF-β containing factor VIII concentrate caused 73% inhibition of proliferation.

As before, equivalent inhibitory amounts of TGF-β1 in purified form and TGF-β containing concentrate were incubated with increasing concentrations of commercial TGF-β neutralising antibody and tested in a T lymphocyte proliferation assay. Profilate SD, when present at 0.25 IU/ml was found to inhibit T lymphocyte

proliferation to the same extent as 50 ng/ml TGF-β1, Figure 38. Higher concentrations of antibody completely neutralised all the inhibitory activity caused by purified TGF-β1, Figure 39. However, the antibody failed to neutralise completely the equivalent activity of Profilate SD, removing only 43% of the inhibitory activity.

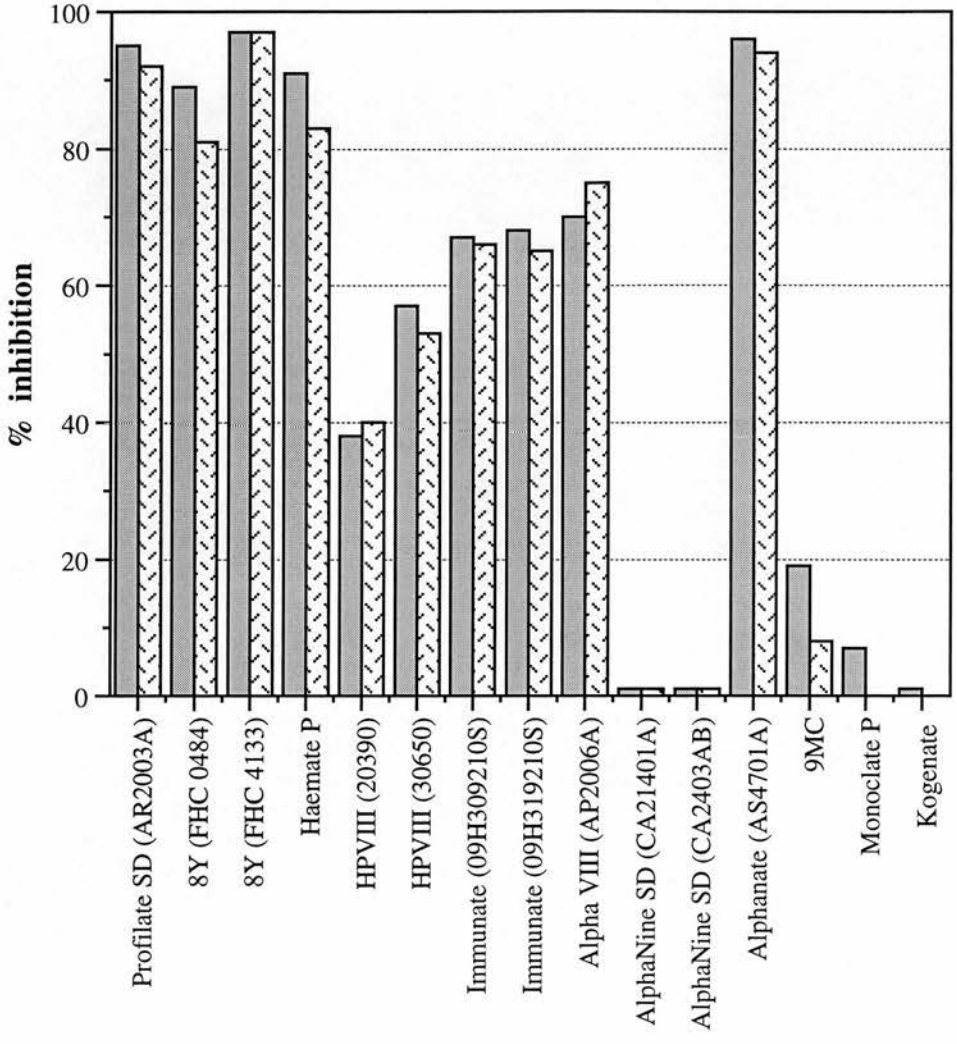


FIGURE 34: Effects of a range of coagulation factor concentrates on PHA-stimulated lymphocyte proliferation in the presence or absence of anti-TGF-β neutralising antibody. Concentrates were present at a final concentration of 1 IU/ml. The antibody failed to abrogate the inhibitory effects of some concentrates on PHA-stimulated lymphocyte proliferation.

coagulation factor concentrate alone

coagulation factor concentrate + TGF-β neutralising antibody

T Lymphocyte Proliferation Assay

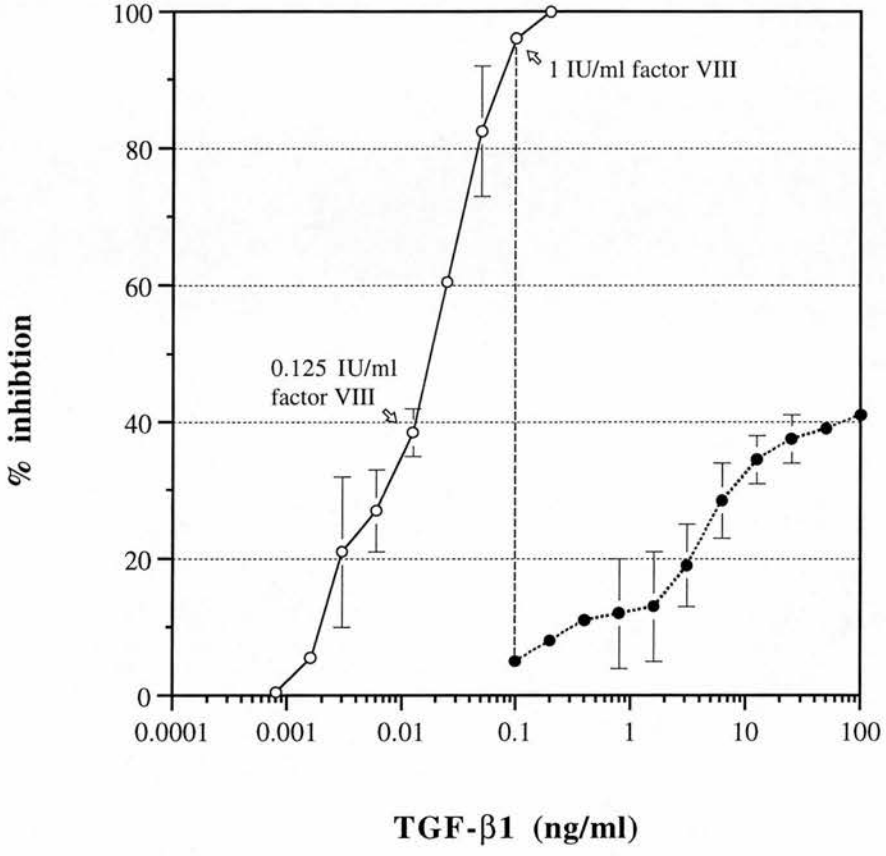


FIGURE 36: Inhibition of proliferation of PHA-stimulated T lymphoctes by the intermediate purity factor VIII concentrate Profilate OSD (batch AR4205A) and purified human TGF-β1. From this experiment it can be seen that 0.125 IU/ml of Profilate OSD and 12.5 ng/ml of purified TGF-β1 are eqivalent inhibitiors of T lymphocyte proliferation. Note that 1 IU/ml of Profilate which contains approximately 0.1 ng/ml of TGF-β1 as assessed by both bioassay and ELISA causes 91% more inhibition than purified human TGF-β1.

—o— Profilate OSD

·····•····· purified human TGF-β1.

T Lymphocyte Proliferation Assay

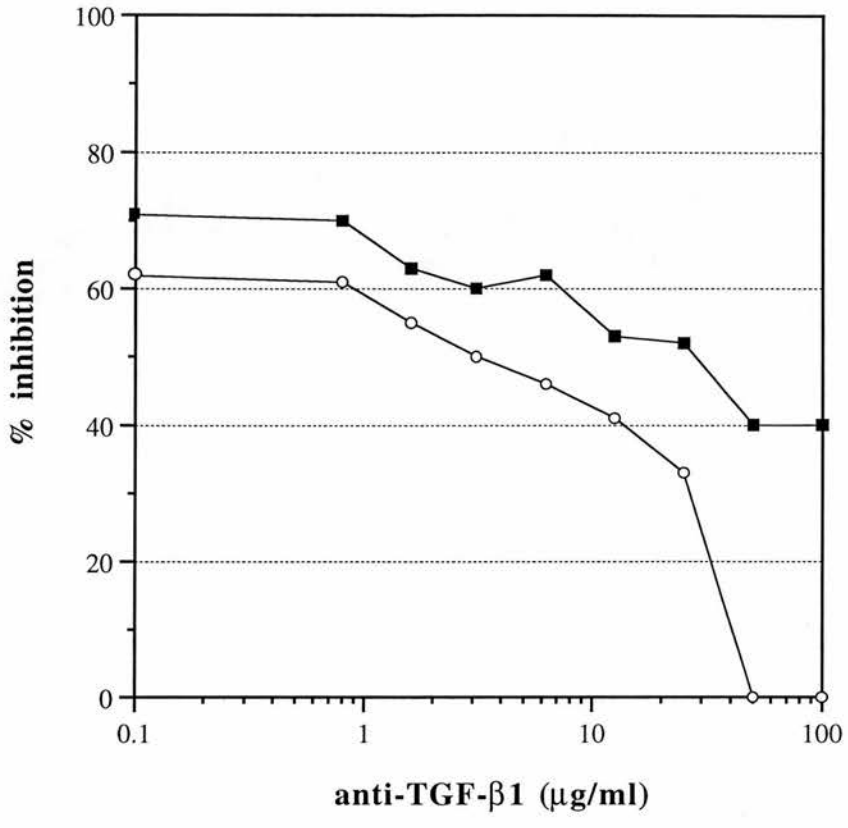


FIGURE 37: Neutralisation of equivalent inhibitory effects of purified human TGF-β1 (12.5 ng/ml) and Profilate OSD (batch AR4205A, 0.125 IU/ml) on the proliferation of PHA-stimulated T lymphocytes.

—■— Profilate OSD

—○— purified human TGF-β1

T Lymphocyte Proliferation Assay

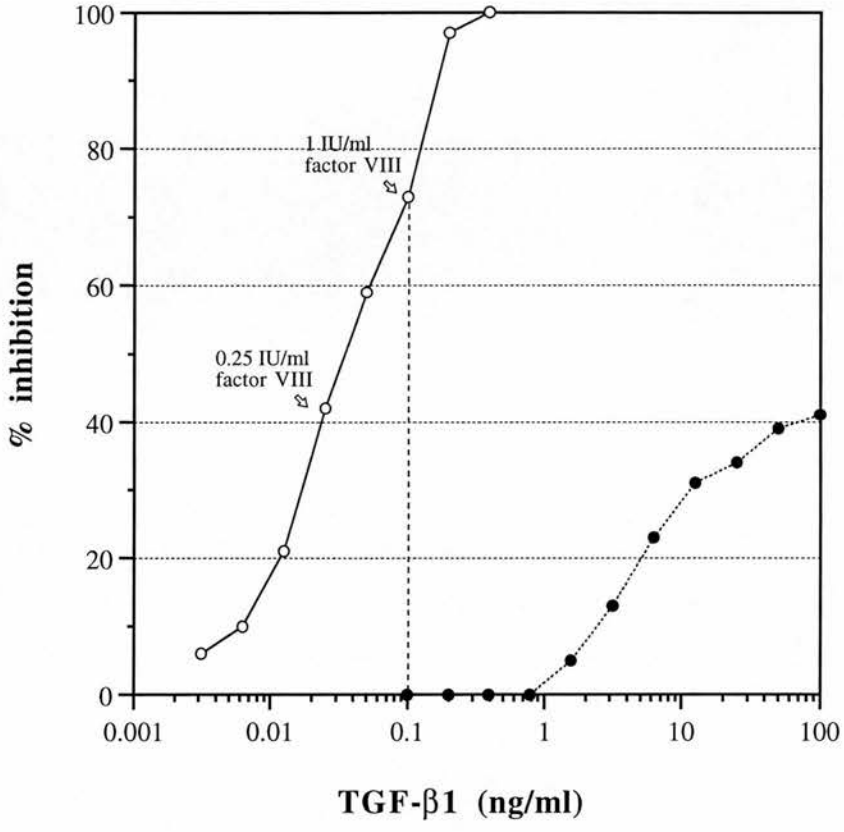


FIGURE 38: Inhibition of proliferation of PHA-stimulated T lymphocytes by the intermediate purity factor VIII concentrate Profilate SD (batch AR2004A) and purified human TGF-β1. From this experiment it can be seen that 0.25 IU/ml of Profilate SD and 50 ng/ml of purified TGF-β1 are equivalent inhibitors of T lymphocyte proliferation.

—o— Profilate SD

·····●····· purified human TGF-β1

T Lymphocyte Proliferation Assay

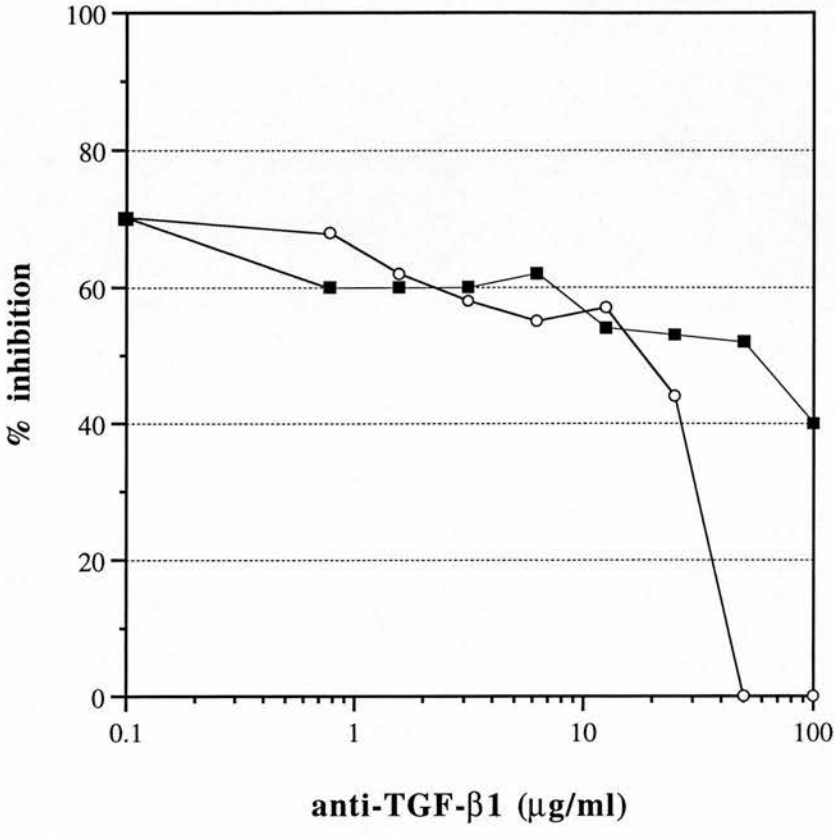


FIGURE 39: Neutralisiation of equivalent inhibitory effects of purified human TGF-β1 (50 ng/ml) and Profilate SD (batch AR2004A, 0.25 IU/ml) on the proliferation of PHA-stimulated T lymphocytes.

—■— Profilate SD (batch AR2004A)

—○— purified human TGF-β1

DISCUSSION

1. Contamination of Coagulation Factor Concentrates with TGF-β.

A report published by Wadhwa *et al* (1994) identified TGF-β as a major contaminant in factor VIII concentrates. Furthermore, the levels of TGF-β detected in factor VIII concentrates were found to correlate strongly with their ability to inhibit IL-2 secretion by a PHA-stimulated T cell line. The authors stated the addition of specific TGF-β antibody reversed the inhibitory effect of some concentrates on IL-2 secretion.

I have measured the levels of biologically active TGF-β and total TGF-β1 in the same sample of coagulation factor concentrates that had previously been assessed for their ability to inhibit the proliferation, IL-2 and IL-6 secretion of PHA-stimulated lymphocytes. Active TGF-β levels were measured by a bioassay which was based on the ability of TGF-β to inhibit the proliferation of the cell line Mv-1-Lu. In agreement with the published results, intermediate purity concentrates and some ion-exchange and affinity purified concentrates inhibited Mv-1-Lu cell proliferation, demonstrating that they contain biologically active TGF-β. Immunoaffinity and recombinant products contained no detectable active TGF-β. Furthermore, in addition to confirming the reported data I have shown that other coagulation factor concentrates, factor IX and von Willebrand factor concentrates of intermediate and ion-exchange purity also contain active TGF-β.

Two factor VIII concentrates Alpha VIII and AlphaNine SD, did not inhibit the proliferation of this cell line, but enhanced its proliferation, suggesting that these concentrates do not contain any active TGF-β. This would agree with the data presented by Wadhwa *et al* who did not detect any biologically active TGF-β in the four batches of Alpha VIII tested. The growth promoting activity present in these two concentrates is probably due to some contaminant(s) which promotes the growth of epithelial cells. It has been documented that the proliferation of Mv-1-Lu cells is also affected by other cytokines, indeed I have shown Mv-1-Lu proliferation is inhibited by both TNF α and IFN α. For this reason, specific neutralising TGF-β

bioassays were carried out on two concentrates which inhibited Mv-1-Lu cell proliferation. The results confirmed that the inhibitory effects that these two concentrates exerted on Mv-1-Lu cell proliferation were indeed due only to the presence of TGF-β. However, due to time constraints, neutralising bioassays were performed on only two concentrates; therefore one can only assume that the inhibition of Mv-1-Lu cells by other concentrates is due to TGF-β contamination.

TGF-β is found circulating in the plasma as a latent complex which is biologically inactive and hence undetectable by bioassay. Therefore, to determine if any latent TGF-β1 was present in coagulation factor concentrates, TGF-β1 was measured by ELISA. Using this method, TGF-β1 was detected in all coagulation factor concentrates tested. Unfortunately, recombinant products were not available for testing in this system. Wadhwa *et al* failed to detect any latent TGF-β in immunoaffinity purified concentrates. However, this could be due to the ELISA technique which I employed being more sensitive than the method used by Wadhwa *et al*, which involved acid activating TGF-β in concentrates, then testing in a bioassay.

As with active TGF-β levels, the levels of total TGF-β1 generally decreased as the product purity increased. The levels of active and total TGF-β measured in each concentrate were found to correlate with its ability to inhibit both the proliferation and IL-2 secretion of PHA-stimulated lymphocytes. However, some exceptions were noted. The factor VIII concentrates Immunate and Alpha VIII which both cause profound inhibition of lymphocyte proliferation contain no active TGF-β, but do contain latent TGF-β1. It may be argued that perhaps the latent TGF-β1 contained in these concentrates is being activated during tissue culture, however, immunoaffinity purified concentrates, which possess no immune modulating activity, contain latent TGF-β1, which is remaining inactive throughout culture. Another exception is the ion-exchange purified factor VIII concentrate HPVIII, which contains active TGF-β but caused only moderate inhibition of proliferation and failed to inhibit IL-2 secretion. These exceptions would suggest that, even though a correlation exists between TGF-β levels and inhibition of lymphocyte function,

TGF-β levels may serve as a marker of concentrate purity rather than being the sole and direct cause of inhibition of lymphocyte function *in vitro*.

In some instances, the active TGF-β measured in concentrates was greater than total TGF-β1 measured. This may be due to the Mv-1-Lu cells used in the TGF-β bioassay being inhibited by other cytokines, or by other members of the TGF-β family, TGF-β2 and TGF-β3, which are not detected by the TGF-β1 ELISA. Since, the polyclonal antibody to TGF-β neutralised all the Mv-1-Lu antiproliferative activity in one such concentrate, HPVIII, it would appear that the increased level of active TGF-β detected is due to other isoforms of TGF-β and not some other contaminant(s). Nonetheless, other contaminants are obviously present in some coagulation factor concentrates since two concentrates did not inhibit the proliferation of Mv-1-Lu cells but enhanced their proliferation.

2. Neutralisation of TGF-β Activity in Coagulation Factor Concentrates

Wadhwa *et al* stated that the addition of a specific TGF-β antibody reversed the inhibitory effects of some concentrates on IL-2 secretion by a PHA-stimulated T cell line. However, upon closer examination of their results, it may be seen that the inhibition of IL-2 secretion caused by seven factor VIII concentrates was reversed by neutralising antibody in only one case, partially reversed (5-50%) in five cases and unaffected in one case. From their results, it can only be concluded that in the majority of factor VIII concentrates tested, contaminating TGF-β is responsible for a portion of the inhibition of IL-2 secretion, and hence is only a component of the immune modulating contaminant, rather than, as suggested, the major contaminant.

Having confirmed that TGF-β was a contaminant of some factor VIII concentrates and having determined that it was also present in other coagulation factor concentrates it remained to be established whether its presence was responsible for the inhibition of lymphocyte function *in vitro*. TGF-β neutralisation was undertaken during lymphocyte proliferation with various concentrates to determine whether TGF-β contamination alone was responsible for inhibiting PHA-stimulated proliferation. TGF-β activity in 1 IU/ml of coagulation factor concentrate was

neutralised by the addition of an antibody to TGF-β1. The antibody used in this initial experiment was the same polyclonal chicken anti-TGF-β antibody used by Wadhwa *et al* in their neutralising assays. When included in PHA-stimulated lymphocyte proliferation assays along with various inhibitory coagulation factor concentrates, the antibody failed to abrogate any significant portion of the observed inhibition of proliferation. A final concentration of 1 IU/ml of coagulation factor concentrates was equivalent to the addition of active TGF-β at a final concentration of 0-0.18 ng/ml. According to data published by Wadhwa *et al*, this antibody when present at the concentration used, was capable of neutralising 5 ng/ml of TGF-β, and hence was used in great excess. The results from this experiment suggests that no portion of the activity inhibiting the proliferation of PHA-stimulated lymphocytes is due to TGF-β.

In order to confirm that the failure of this antibody to reverse inhibition of proliferation was not due to loss of activity of the donated antibody, a commercial neutralising polyclonal antibody was included in a similar PHA-stimulated lymphocyte proliferation assay. Even though this commercial antibody was present at a concentration capable of neutralising 7.5 ng/ml of TGF-β1, it failed to neutralise the lymphocyte proliferation inhibitory activity of three factor VIII concentrates. This design of neutralising experiment also carried out by Wadhwa *et al* did not include controls such as purified TGF-β1 alone and purified TGF-β1 plus antibody, to ensure that the antibody was neutralising TGF-β. Therefore, a second neutralising strategy was devised whereby equivalent amounts of control purified TGF-β1 and of TGF-β contained in a factor VIII concentrate were incubated with increasing concentrations of the commercial neutralising antibody, prior to the addition of PHA-stimulated lymphocytes. When factor VIII concentrates were present at what was equivalent (in terms of functional effect) to 1 ng/ml active TGF-β, the antibody, even at very high concentrations, did not neutralise the inhibitory activity in the two concentrates. From this set of experiments, it can again be concluded, that even though active TGF-β is present in coagulation factor concentrates, its presence does not account for any of the observed inhibition of PHA-stimulated lymphocyte proliferation.

Contaminating TGF-β in coagulation factor concentrates was also targeted for removal by immunoprecipitation. This strategy proved to be unsuccessful, since when concentrates known to contain active TGF-β were immunoprecipitated for TGF-β, no change in the concentrates' activity in TGF-β bioassay was observed.

As mentioned above, as a control for these neutralising experiments, purified human TGF-β1 was incubated with PHA-stimulated lymphocytes. Addition of up to 100 ng/ml of purified TGF-β failed to inhibit the proliferation of PHA-stimulated lymphocytes, suggesting that lymphocytes are not responding to the addition of exogenous TGF-β in purified form or contained in coagulation factor concentrates. It is possible that the response of PHA-stimulated lymphocytes to purified TGF-β1 may be blunted due to saturation with TGF-β already contained in FCS. Purified TGF-β1 was found to have slight inhibitory effects on lymphocyte proliferation in the presence of two of the batches tested but this inhibition did not reach statistically significant levels. Following this result, the final percentage of FCS used was varied to determine if a reduction would permit the detection of inhibition by exogenous purified TGF-β1. A reduction in the percentage of FCS from 10% to 2% brought about a further small relative increase in the measurable effect of purified TGF-β1 on PHA-stimulated lymphocytes. However, the inhibition of proliferation was still not statistically significant. This lack of inhibition of lymphocyte proliferation by purified TGF-β1 may also be due to α_2M also contained in FCS. As discussed previously, the serum protein α_2M binds bioavailable TGF-β, forming a biologically latent complex. It is perhaps this complex formation which accounts for lack of inhibition of proliferation of lymphocytes on the addition of purified TGF-β1.

Purified TGF-β1 has been demonstrated to inhibit the proliferation of PHA-stimulated purified T lymphocytes (Ahuja *et al*, 1993; Fox *et al*, 1992). Therefore, purified T lymphocyte preparations were substituted for unseparated lymphocytes in PHA-stimulated proliferation assays. As reported, purified TGF-β1 did indeed inhibit T lymphocyte proliferation in PHA-stimulated tests. Following this result two TGF-β containing intermediate purity factor VIII concentrates were tested in a PHA-stimulated T lymphocyte proliferation assay in parallel with an equivalent amount of purified human TGF-β1. However, addition of equivalent amounts of

purified TGF-β1 and that contained in the factor VIII concentrates did not inhibit T lymphocyte proliferation to the same extent. When present at 0.1 ng/ml in purified form, TGF-β caused only slight or no inhibition, whereas, when present at the same concentration in the two factor VIII concentrates, almost complete inhibition was observed. This result would seem to suggest that contaminant(s) present in these concentrates other than TGF-β1 are responsible for the majority of the T lymphocyte inhibitory activity.

To determine what portion of the T lymphocyte inhibitory activity was due to TGF-β contamination with biologically active TGF-β neutralising T lymphocyte proliferation assays were carried out on these concentrates. The results obtained suggest that approximately half of the T lymphocyte inhibitory activity of these two intermediate purity concentrates is due to TGF-β contamination.

In conclusion, while TGF-β contamination is not responsible for any of the observed inhibition of PHA-stimulated peripheral blood lymphocyte proliferation caused by concentrates, it is responsible for 43-44% of their inhibitory effects on purified T lymphocyte proliferation.

3. Impact of Levels of Contaminating TGF-β in Coagulation Factor Concentrates on Plasma TGF-β Levels

Total TGF-β1 levels were measured in the plasma and serum of normal individuals, and the increase in the level of TGF-β1 calculated if that individual were to be transfused with a concentrate containing relatively high levels of contaminating TGF-β1. If an individual (with a basal level of 41 ng/ml total TGF-β1) were to receive 1500 ng of TGF-β1 (equivalent to 3000 IU of coagulation factor) this would lead to an increase of only 0.5 ng/ml in total plasma TGF-β1. It seems inconceivable that the very small increase would have any impact on the immune system of an individual whose normal plasma TGF-β1 level is in the order of 41 ng/ml. Therefore, it may be concluded that TGF-β is probably not responsible for immune modulation observed in HIV-negative haemophiliacs.

CHAPTER 5

Studies on the Molecular Nature of the Immunosuppressive Agent(s) in Factor VIII Concentrates

INTRODUCTION

The nature of the conatminant(s) responsible for inhibiting PHA-stimulated lymphocyte proliferation was invsetigated further, since no specific contaminant had been implicated as being responsible for the inhibition of PHA-stimulated lymphocyte proliferation. Several approaches as outlined in this chapter, were taken in order to attempt to identify contaminants of immunomodulatory behaviour.

The presence of proteins in factor VIII concentrates other than factor VIII itself was investigated by two methods. Firstly the components of a selection of concentrates were separated electrophoretically by denaturing SDS-polyacrylamide gel electrophoresis, and the proteins visualised by silver stainning. The results demonstrated the presence of many proteins in the concentrates that were not to factor VIII.

Confirmation of the presence of contaminating proteins lead to the design of an experiment to investigate whether the inhibitory activity of a concentrate was entirely due to protein contamination or a contaminant of some other nature. This investigation involved a concentrate being exposed to the protease trypsin, and being tested in a lymphocyte proliferation assay. The results from this crude experiment suggested that only a portion of the activity of this concentrate was due to protein contamination.

Many entries in the literature have suggested that the presence of a low molecular weight dialysable component may influence lymphocyte proliferation (McDonald *et al*, 1985; Vermot Desroches *et al*, 1992; Wadhwa *et al*, 1992). Accordingly, dialysis of several inhibitory products was carried out. The results showing that some products purified by ion-exchange and affinity chromatography contained a dialysable inhibitory substance.

As these preliminary experiments only provided sketchy details as to the nature of the contaminants, gel filtration of a factor VIII concentrate was performed, in an attempt to isolate particular components and estimate their molecular weight. Fractionation revealed the presence of six lymphocyte inhibitory activities, two of which were identified, one containing TGF-β the other containing formulation buffer. Unfortunately, the four other activities remain unidentified.

MATERIALS AND METHODS

1. Polyacrylamide Gel Electrophoresis

1.1 Sample Preparation

Factor VIII concentrates were reconstituted according to manufacturers' instructions and 4 µl (final concentration of approximately 2 µg protein) added to 11 µl of 1 x SDS gel-loading buffer (0.05 M Tris-Cl (pH 6.8), 0.1 M dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol). Dithiothreitol (DTT) (Sigma-Aldrich Ltd.) was added to the loading buffer from a 1 M stock just before use.

Preparations of human albumin (20% solution. Immuno Ltd, UK) and purified human TGF-β1 were also prepared to be electrophoresed. 4 µl aliquots of human albumin solution and of 2 µg/ml of purified human TGF-β1 (R & D Systems Ltd.) were added to 11 µl of 1 x SDS gel-loading buffer.

Rainbow molecular weight markers (Amersham Life Science) high molecular weight range (14.3-200 kDa) or low molecular weight range (2.4-46 kDa) were also prepared by adding 10 µl of marker to 5 µl of 1 x SDS gel-loading buffer. All samples were denatured by heating to 100°C for 3 minutes prior to loading.

1.2 SDS-PAGE

Electrophoresis of samples was performed under denaturing conditions using slab gels in the discontinuous buffer system of Laemmli (1970). Samples were resolved by electrophoresis through 6, 8, 10 and 15% polyacrylamide gels, containing 0.375 M Tris (pH 8.8) and 0.1 % SDS.

A volume of 30 ml of resolving gel mix was polymerised following the addition of 25 µl N,N,N',N'-tetramethylethylenediamine (TEMED) and 300 µl ammonium persulphate (both supplied by Sigma-Aldrich Ltd.).

A 5% polyacrylamide stacking gel containing 0.25 M Tris (pH 6.8) and 0.1% SDS was used to concentrate the sample before resolution. A volume of 10 ml of stacking gel mix was prepared and poured on top of each resolving gel.

Samples were electrophoresed for approximatley 4 hours at voltage of 15 V/cm in Tris-glycine electrophoresis buffer containing 0.025M Tris, 0.25 M glycine and 0.1% SDS.

1.3 Silver Staining

SDS-polyacrylamide gels were silver stained using the Silver Stain Plus Kit (Biorad Laboratories Ltd. UK). This kit allowed the detection of nanogram quantities of protein. Staining was carried out according to manufacturers' instructions.

Gels were fixed in fixative enhancer solution with gentle agitation for 30 minutes at room temperature. The fixative enhancer solution was decanted from the staining vessel and the gels washed twice by gentle agitation in deionised distilled water for 20 minutes. Gels were stained and developed in a freshly prepared staining and developing solution for 20 minutes or until desired staining intensity was reached. Following staining, the stain was discarded and the gels placed in a 5% acetic acid (BDH Laboratory Supplies Ltd, UK) solution to stop the staining reaction. After stopping the reaction, the gels were rinsed in distilled water for 5 minutes, then photographed.

2. Physical Manipulation of Coagulation Factor Concentrates

2.1. Immunoprecipitation of TGF-β

TGF-β present in coagulation factor concentrates was targeted for removal by immunoprecipitation. This procedure involved the binding of antibody to TGF-β to an insoluble matrix. The product for this purpose used was GammaBind G Plus Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden). GammaBind G is a recombinant form of Protein G, immobilised to Sepharose CL-6B, which binds to the F_c region of IgG from a variety of species, leaving the F_{ab} region available for binding antigen. One ml aliquots of concentrate were incubated on ice for 1 hour with 100 µg of commercial anti-TGF-β antibody, prior to the addition of 20 µl of a

50% slurry of GammaBind G Plus Sepharose in PBS. The beads were rotated for even mixing for 4 hours at 4°C. The Sepharose was removed by brief microfugation at high speed and the supernatant retained. A further 100 μg of antibody was added to the supernatant and the procedure repeated. The depleted coagulation factor concentrates were sterile filtered (μStar. Pore diameter 0.2 μm) and tested immediately in PHA-stimulated lymphocyte proliferation assays and TGF-β bioassays, as described previously on pages 88 and 125 respectively.

2.2 Trypsinisation of a Coagulation Factor Concentrate

For this purpose, serial doubling dilutions of trypsin (0.08-1 mg/ml) (Bovine Pancreas. Sima-Aldrich Company) in 25 μl volumes were prepared in triplicate in 96-well flat-bottomed-plates. To each trypsin dilution 2 IU/ml of Profilate OSD was added. Plates were incubated at 37°C for 1 hour before the addition of trypsin inhibitor (0.02- 3 mg/ml) (Soya bean. Sigma-Aldrich Company Ltd). In order to neutralise completely the trypsin activity, three times the amount of trypsin inhibitor to trypsin was added. The resulting mix of 100 μl volume was incubated for a further hour at 37°C before the addition of PBMC. A PBMC suspension containing $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FCS was prepared and 100 μl added to each well. PHA was included at a final concentration of 1 μg/ml. Control cultures of PBMC in culture medium alone, PBMC and PHA alone, and PBMC plus Profilate OSD in the absence of PHA were included in the assay. Cultures were incubated for 96 hours before harvesting. Proliferation was measured by pulsing each well with 1μCi of [methyl-^{3}H]thymidine 18 hours before cell harvesting.

2.3 Dialysis of Clotting Factor Concentrates

Benzoylated cellulose dialysis tubing (Sigma-Aldrich Ltd.) was used to remove microsolutes of less than 2 kDa from clotting factor concentrates. Concentrates were reconstituted according to manufacturers' instructions and 5 ml placed in dialysis tubing that was tightly tied to expel air. Concentrates were dialysed against RPMI 1640 medium (no antibiotics or FCS included) overnight at 4°C with three changes of solution. Following dialysis, the resulting solution was sterile filtered (μStar. Pore diameter 0.2 μm). A 1 ml aliquot of each concentrate was retained, and stored at 4°C as a control. A further 1 ml aliquot of concentrate was also retained and sterile filtered as a control. Dialysed concentrates were tested in a PHA-stimulated lymphocyte proliferation assays as described previously on page 88.

3. Gel Filtration

3.1 Column Dimensions

Two columns were used to fractionate the ion-exchange factor VIII concentrate HPVIII.

Column 1:- volume 300 ml, height 40 cm, radius 1.5 cm, packed with Sepharose CL-4B (molecular weight fractionation range 60 - 2000 kDa. Pharmacia LKB Biotechnology).

Column 2:- 180 ml volume, height 90 cm, radius 0.8 cm, packed with Sephadex G-150 (molecular weight fractionation range 5 - 300 kDa. Pharmacia LKB Biotechnology).

3.2 Preparation of Column Media

Sepharose CL-4B was supplied as a ready-to-use suspension containing 20% ethanol as a preservative. Following packing of the column, three column volumes of PBS were passed through in order to stabilise the bed and remove the ethanol. Sephadex G-150 was prepared by mixing 10 grams of powdered G-150 with 300 ml of PBS. The resulting thick slurry was swollen in a boiling water bath for 5 hours, then allowed to reach the temperature of column operation before packing. Once the column was packed, three column volumes of PBS were passed through the column.

3.3 Column Running Conditions

Column 1 was eluted with PBS at a rate of 20 ml/hour. A total of 80 fractions of a 4 ml volume were collected. Fractionation was carried out at room temperature (18-22°C).

Column 2 was eluted with PBS at a rate of 10 ml/hour. A total of 200 fractions of a 1 ml volume were collected. Fractionation was carried out at 4°C.

3.4 Void Volume Determination

The void volume (Vo) of the columns was determined by running a solution of Blue Dextran 2000 (molecular weight 2000 kDa. Pharmacia LKB Biotechnology) down the columns. A 1 mg/ml solution of Blue Dextran in PBS was freshly prepared, filtered and applied to the column. The volume of the blue dextran loaded was 1-2% of the total gel bed volume. The presence of Blue Dextran in the fractions was monitored at a wavelength of 620 nm. The Vo was calculated by measuring the volume of eluent from the start of sample application to the centre of the Blue Dextran elution peak.

3.5 Calibration of Gel Filtration Columns

Calibration of column 1 was performed by running 0.5 ml of normal human serum (prepared as described previously) diluted with 2.5 ml of PBS down the column and collecting 80 fractions of a 4 ml volume. Fractions were assayed for 1gM (970 kDa) and IgG (146 kDa) by radial immunodiffusion (RID) and for albumin (68 kDa) by absorption at 280 nm.

Calibration of column 2 was performed in two stages, in order that the proteins be sufficiently resolved. One ml of human serum (prepared as described previously) diluted in 1 ml of PBS, was fractionated and the 200 fractions collected, assayed for IgM and IgG by RID, and for albumin by absorption at 280 nm. Following this, 2 ml of a solution containing a final concentration of 1 mg/ml lysozyme (14.3 kDa) (Sigma-Aldrich Ltd.) and 1 mg/ml aprotinin (6.5 kDa) (Sigma-Aldrich Ltd.) was passed through the column and the fractions monitored for the presence of protein by absorption at 280 nm.

3.6 Radial Immunodiffusion Plates

RID plates were prepared by dissolving 0.075 grams of purified agar (Difco Laboratories, USA) in 5 ml of PBS. The agar solution was warmed until boiling, then allowed to cool to 37°C before either 200 µl of anti-human IgM serum (mu-chain specific. S.A.P.U.) or 125 µl of anti-human IgG serum (gamma-chain specific. S.A.P.U) was added. The resulting mix was poured into 5 cm diameter Petri dish (Sterilin. Mackay and Lynn, UK) and allowed to cool to room temperature. Once set, seven equidistant wells of 2 mm diameter were bored in the agar. Plates were stored at 4°C until required.

In order to assay for the presence of immunoglobulins, 2 µl of each fraction were pipetted into the RID plate wells, and plates left to stand at room temperature. Appropriate controls of purified IgG or IgM (S.A.P.U.) were included in every plate. Plates were read on an RID plate reader (Binding Site Ltd, UK) after 18 hours for IgG plates and 48 hours for IgM plates.

3.7 Sample Preparation and Application

The factor VIII concentrate HPVIII, batch number 30540, was fractionated on column 1. This batch of HPVIII contained 230 IU/vial of factor VIII and approximately 5 mg/vial plasma proteins. A total of 460 IU of HPVIII 30540 in 4 ml of distilled water was applied to column 1.

The factor VIII concentrate HPVIII, batch number 30650, was fractionated on column 2. This batch of HPVIII contained 315 IU/vial of factor VIII and approximately 5 mg/vial plasma proteins. A total of 315 IU of HPVIII 30650 in 2 ml of distilled water was applied to column 2.

A 2 ml volume of formulation buffer used in the manufacture of HPVIII was also fractionated on column 2. The formulation buffer contains sodium citrate (3 mg/ml), calcium chloride (0.15 mg/ml), sodium chloride (15 mg/ml), glycine (9 mg/ml), lysine (3 mg/ml) and sucrose (1 mg/ml).

3.8 Testing of Column Fractions

Column fractions were monitored for protein by measuring their absorbance at 280 nm.

Gel filtration fractions were tested in lymphocyte proliferation assays (final PHA concentration 1 μg/ml) and TGF-β bioassays for inhibitory activity. For assay, triplicate wells containing 50 μl of fraction, 50 μl of culture medium and 100 μl of lymphocyte or Mv-1-Lu cell suspension (cell concentration as stated previously) were prepared. Culture conditions, harvest and pulse time were as described previously. For details of both assays see pages 88 and 125 respectively. All fractions were sterile filtered (μStar. Pore diameter 0.2 μm) before being included in assays.

In order to detrmine the molecular weight of the proteins of interest, the elution volume was measured and the calibration curve for each column used to determine the molecular weight of the unknown.

RESULTS

1. Characterisation of Inhibitory Component(s) Present in Coagulation Factor Concentrates

1.1 SDS PAGE of Factor VIII Concentrates

SDS polyacrylamide gel electrophoresis was used to visualise the protein composition of factor VIII concentrates. Four factor VIII concentrates of varying purity, purified human TGF-β1 and purified human albumin were separated by electrophoresis, through 6, 8, 10 and 15% SDS polyacrylamide gels, Figures 40-43, respectively. The gels were silver stained using a commercial kit which allowed the detection of nanogram quantities of protein.

The 6, 8 and 10% gels resolved the high molecular weight components of these preparations, and the 15% gel the low molecular weight components. Purified human albumin was included as a control, since it was added as a carrier protein during the reconstitution of purified human TGF-β1. In addition, both the affinity purified product and the monoclonally purified product have human albumin added as a stabiliser. Inclusion of these controls allowed the identification of protein bands belonging to albumin or contaminating TGF-β in these preparations.

Many bands with both high and low molecular weights were observed in all factor VIII concentrates, Figures 40-43. Some bands were of very low intensity, suggesting that these proteins are present in only trace amounts.

The 6% polyacrylamide gel allowed separation of components between 46 and 200 kDa, Figure 40. The multiple polypeptides of factor VIII which have a molecular weight range of 80-210 kDa, were not visible on this gel, perhaps due to the minute amounts present. A band with a molecular weight >200kDa was identified which appeared only in the lymphocyte inhibitory concentrates. No other bands appearing exclusively in the inhibitory concentrates were identified.

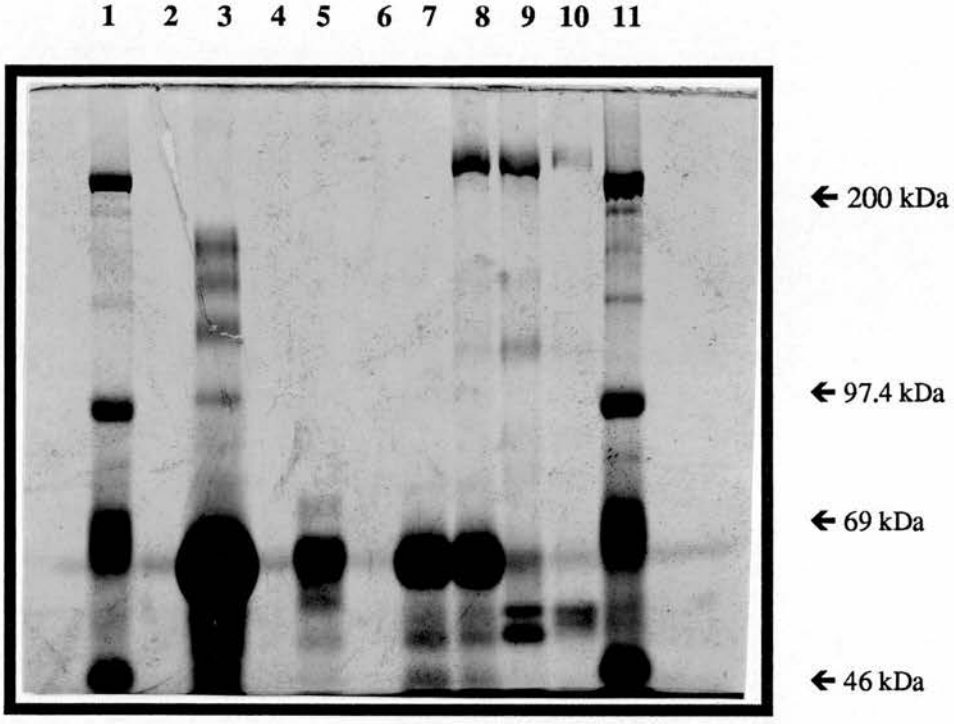
The 8% polyacrylamide gel allowed separation of components between 30 and 200 kDa, Figure 41. Multiple protein bands were observed in all the preparations tested.

Many of these bands in the immunoaffinity and affinity purified products could be attributed to albumin and degradation products of albumin. Other protein bands with a molecular weight of 80-210 kDa could be attributed to factor VIII polypeptides. A band with molecular weight >200 kDa was again observed only in those concentrates which were inhibitory to lymphocyte function.

The 10% polyacrylamide gel allowed separation of components between 21.5 and 200 kDa, Figure 42. The band at >200 kDa was again observed only in inhibitory concentrates. This band appeared less intense in HPVIII than in the other two concentrates, Profilate OSD and Alphanate which inhibit lymphocyte proliferation to a greater extent than HPVIII.

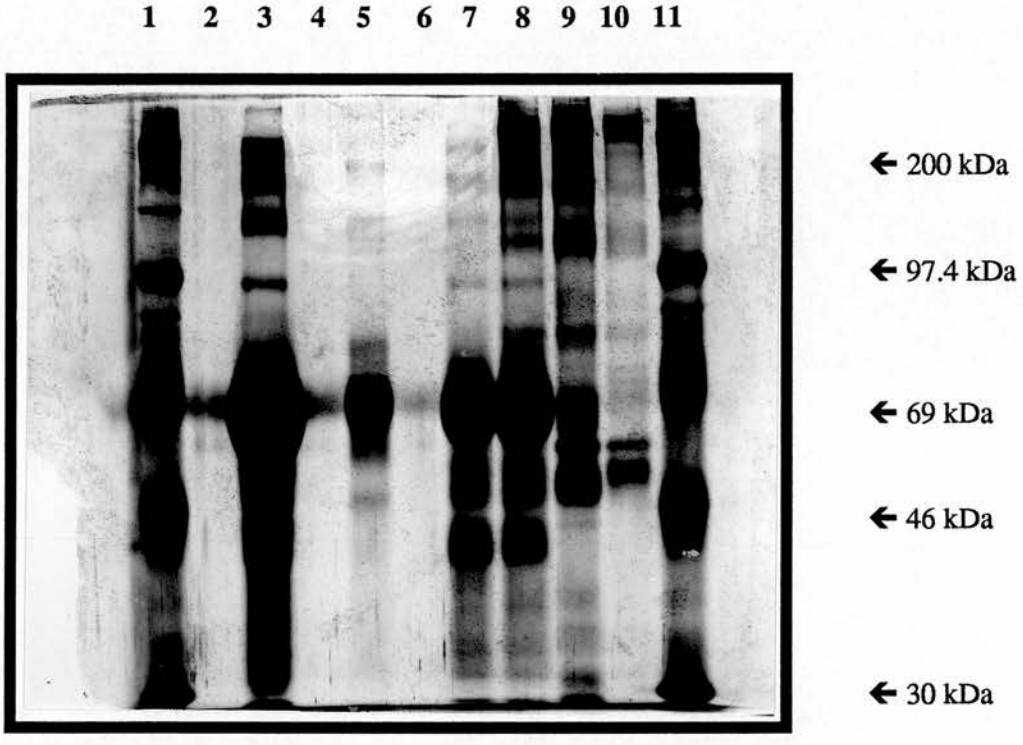
The 15% polyacrylamide gel allowed separation of components between 2 and 46 kDa, Figure 43. Bands of the correct molecular weight for TGF-β1 (25 kDa homodimer, and 12.5 kDa monomer) were not observed when purified human TGF-β1 was resolved on polyacrylaimde gels.

FIGURE 40: 6% SDS POLYACRYLAMIDE GEL



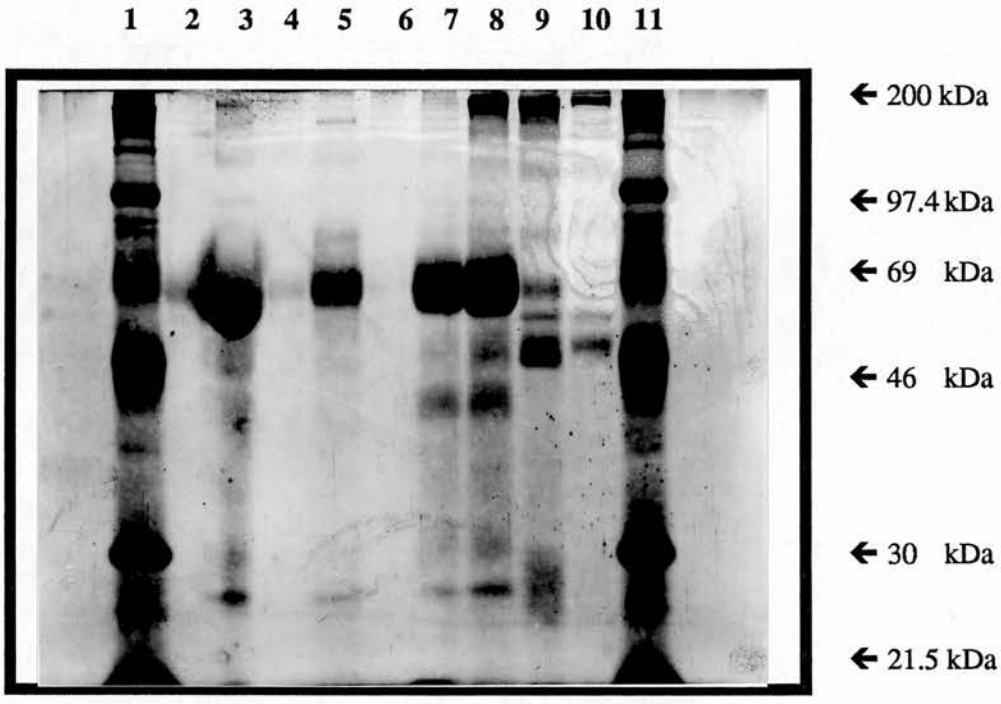
Lane Number	Sample
1	HMW marker
2	blank
3	human albumin (Immuno)
4	blank
5	purified human TGF-β1 (Genzyme)
6	blank
7	Monoclate P (batch number J53706)
8	Alphanate (batch number AS4701A)
9	Profilate SD (batch number AR2003A)
10	HPVIII (batch number 40820)
11	HMW marker

FIGURE 41: 8% SDS POLYACRYLAMIDE GEL



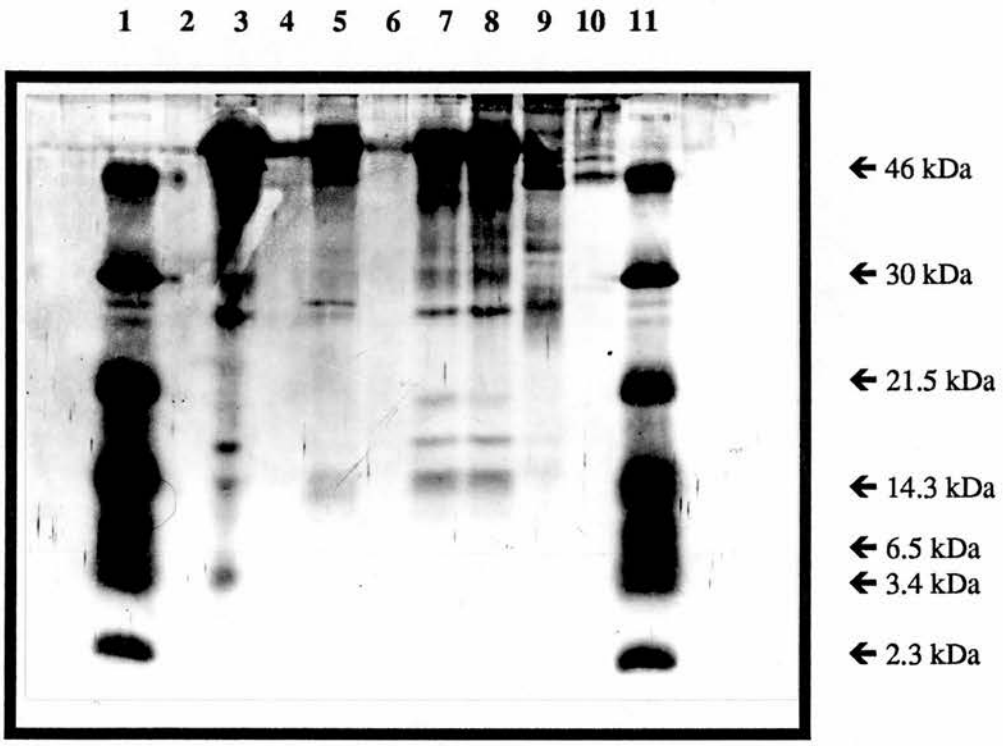
Lane Number	Sample
1	HMW markers
2	blank
3	human albumin (Immuno)
4	blank
5	purified human TGF-β1 (Genzyme)
6	blank
7	Monoclate P (batch number J53706)
8	Alphanate (batch number AS4701A)
9	Profilate SD (batch number AR2003A)
10	HPVIII (batch number 40820)
11	HMW markers

FIGURE 42: 10% SDS POLYACRYLAMIDE GEL



Lane Number	Sample
1	HMW markers
2	blank
3	human albumin (Immuno)
4	blank
5	purified human TGF-β1 (Genzyme)
6	blank
7	Monoclate P (batch number J53706)
8	Alphanate (batch number AS4701A)
9	Profilate SD (batch number AR2003A)
10	HPVIII (batch number 40820)
11	HMW markers

FIGURE 43: 15% SDS POLYACRYLAMIDE GEL



Lane Number	Sample
1	LMW markers
2	blank
3	human albumin (Immuno)
4	blank
5	purified human TGF-β1 (Genzyme)
6	blank
7	Monoclate P (batch number J53706)
8	Alphanate (batch number AS4701A)
9	Profilate SD (batch number AR2003A)
10	HPVIII (batch number 40820)
11	LMW markers

1.2 Trypsinisation of a Factor VIII Concentrate

To assess whether all the inhibitory activity of coagulation factor concentrates was due to the presence of protein, the intermediate purity factor VIII concentrate Profilate OSD was treated with trypsin and included in a lymphocyte proliferation assay. Profilate OSD was incubated at 37°C for 1 hour with increasing concentrations of trypsin (0.08 -1 mg/ml). The trypsin activity was then neutralised by the addition of trypsin inhibitor (0.02 -3 mg/ml) and further incubation for 1 hour. A semilogarithmic graph was constructed of log concentration of trypsin : trypsin inhibitor (0.03 - 4 mg/ml) versus percentage inhibition of lymphocyte proliferation, Figure 45.

As shown previously, Profilate OSD, when present at 2 IU/ml, caused 80% inhibition of lymphocyte proliferation, Figure 9. The lowest concentration of trypsin had no effect on the inhibitory activity of Profilate OSD. However, as the trypsin concentration increased, the percentage inhibition of lymphocyte proliferation decreased until 0.5 mg/ml trypsin : trypsin inhibitor complex was reached. At this concentration and higher, the percentage inhibition caused by Profilte OSD reached a plateau at approximately 60%. Higher concentrations of trypsin : trypsin inhibitor complex than 0.5 mg/ml were found to inhibit lymphocyte proliferation.

If the inhibition caused by these higher levels of trypsin : trypsin inhibitor complex was subtracted from the percentage inhibition of proliferation due to trypsinised Profilate OSD, it can be seen that the highest concentration of trypsin is capable of removing approximately 59% of the observed inhibitory activity of 2 IU/ml Profilate OSD, leaving a total of 41% inhibition which is trypsin resistant.

Lymphocyte Proliferation Assay

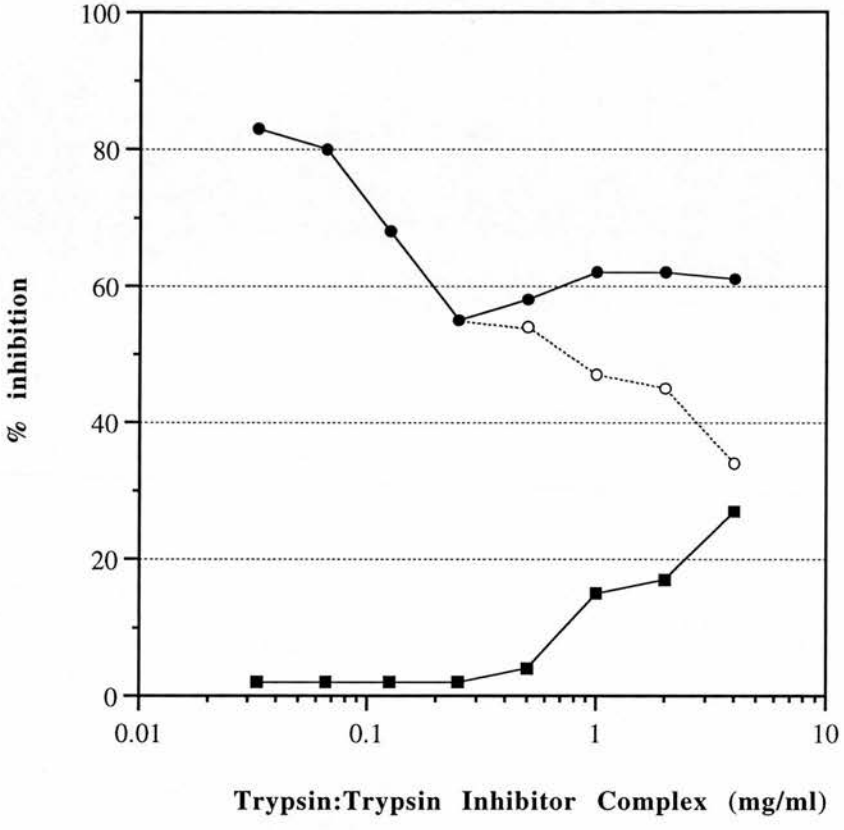


FIGURE 44: Removal of lymphocyte inhibitory activity of Profilate OSD (batch AR4205A) by trypsinisation.

—●— Trypsin treated Profilate OSD (2IU/ml)

—■— Trypsin:Trypsin Inhibitor Complex

·····○····· Trypsin treated Profilate OSD (2IU/ml) minus inhibitory effect of Trypsin:Trypsin Inhibitor Complex

1.3 Dialysis of Factor VIII Concentrates

To determine if any portion of lymphocyte proliferation inhibitory activity was due to some dialysable, low molecular component of coagulation factor concentrates, inhibitory concentrates were dialysed against RPMI 1640 culture medium, at 4°C overnight.

Following dialysis, concentrates were sterile filtered, before testing. In some cases, to ensure no activity was lost in the filtration process, undialysed concentrate was filtered as a control. Doubling dilutions of all manipulations were tested in PHA-stimulated lymphocyte proliferation assays.

Dialysis of the intermediate purity factor VIII concentrate Prolfilate OSD, had no effect on its ability to inhibit lymphocyte proliferation, Figure 46. Filtration of the concentrate through a 0.2 μm filter also had no effect on the inhibitory activity.

In contrast, dialysis of ion-exchange purified factor VIII concentrate, HPVIII and the affinity purified factor VIII concentrate Alpha VIII removed approximately 46% and 41% respectively of the inhibitory activity, Figures 47 and 48.

Dialysis of the inhibitory affinity purified factor VIII concentrate Alphanate was found to remove approximately 25% of the inhibitory activity, Figure 49.

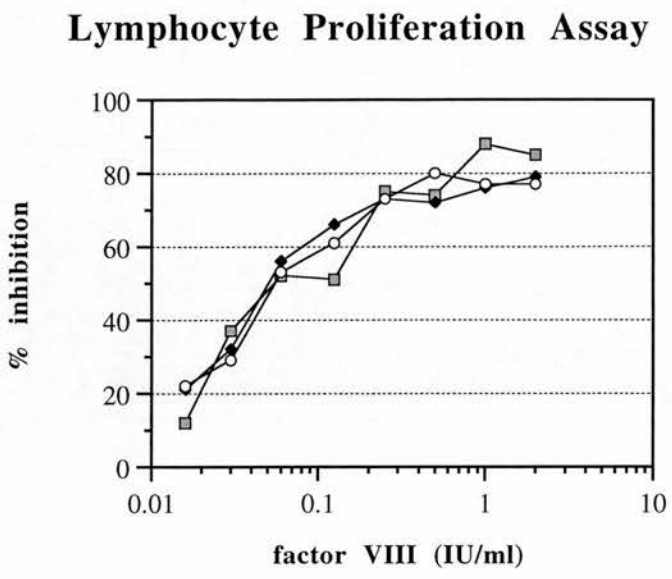


FIGURE 45: Inhibition of proliferation of PHA-stimulated lymphocytes by the intermediate purity factor VIII concentrate Profilate OSD (batch AR4205A), and various physical manipulations of this concentrate.

—○— Profilate OSD —◆— filtered Profilate OSD

—□— dialysed Profilate OSD

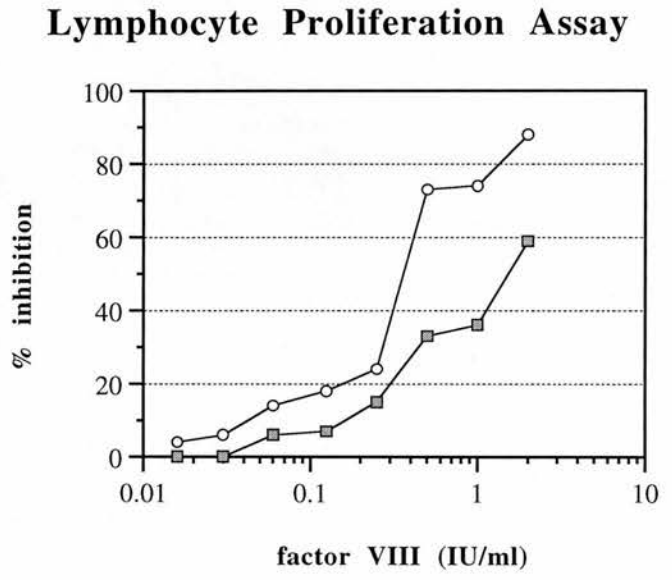


FIGURE 46: Inhibition of PHA-stimulated proliferation of lymphocytes by the ion-exchange purified factor VIII concentrate HPVIII, and dialysed HPVIII.

—○— HPVIII control —□— dialysed HPVIII

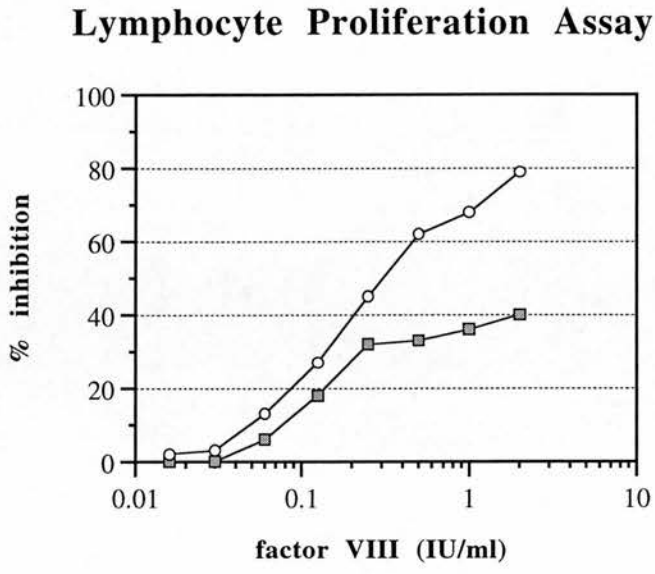


FIGURE 47: Inhibition of proliferation of PHA-stimulated lymphocytes by the affinity purified factor VIII concentrate Alpha VIII (batch AP2005A), and various physical manipulations of the concentrate.

Alpha VIII control — dialysed Alpha VIII

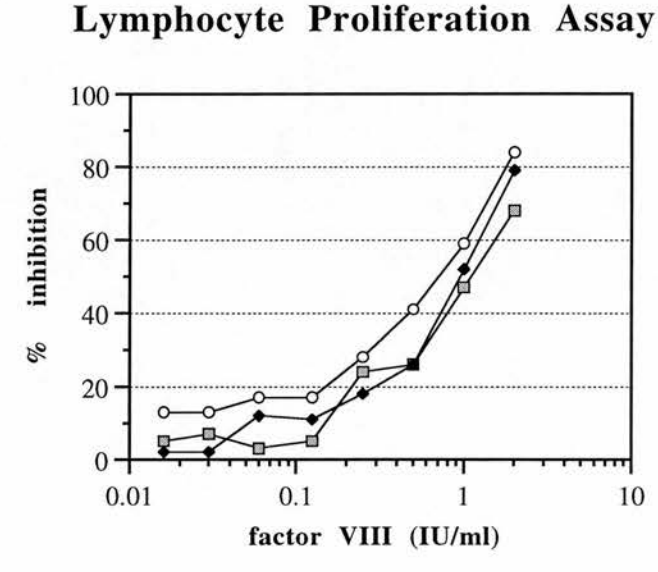


FIGURE 48: Inhibition of proliferation of PHA-stimulated lymphocytes by the affinity purified factor VIII concentrate Alphanate (batch AS4701A), and various physical manipulations of this concentrate.

Alphanate control — filtered Alphanate

dialysed Alphanate

1.4. Fractionation of a Factor VIII Concentrate by Gel Filtration

Gel filtration of a factor VIII concentrate enabled the separation of components of that concentrate according to differences in size. The concentrate chosen for this purpose was the ion-exchange purified product HPVIII. Fractions collected from gel filtration columns were monitored for the presence of protein by absorption at 280 nm. Fractions were also tested in triplicate for inhibitory activity in lymphocyte proliferation assays or in a TGF-β bioassay.

Two batches of the concentrate HPVIII were fractionated, batches 30540 and 30650. Both these batches of HPVIII had previously been shown to inhibit lymphocyte proliferation, causing 70% and 67% inhibition of lymphocyte proliferation respectively, when present at 1 IU/ml. Both batches also contained active TGF-β as assessed by bioassay; 30540 contained 2.53 ng/ml and 30650 contained 1.44 ng/ml.

Results from gel filtration experiments are expressed in the form of an elution diagram, where elution volume is plotted against absorbance (280 nm), and percentage inhibition of lymphocyte proliferation or percentage inhibition of Mv-1-Lu cell proliferation.

(i) Fractionation through Column 1

Column 1 was packed with the gel filtration media Sepharose CL 4B, which has a fractionation range of 60-2000 kDa for globular proteins. Prior to fractionation of the concentrate, the Vo (void volume) of the column was determined and the column calibrated by fractionating standards of known molecular weight. The Vo of the column was found to be 84 ml. A calibration curve of elution volume versus log molecular weight of standards for column 1 was constructed and is shown in Figure 49. A total of 460 IU/ml (10 mg of protein) of HPVIII batch 30540, was applied to the column and 80 fractions of a 4 ml volume collected.

Two protein peaks were detected by absorbance at 280 nm, the first with an elution volume of 144 ml, the second at 284 ml, Figure 50. Those fractions monitored for protein were then sterile filtered, and assayed both for their ability to inhibit PHA-stimulated lymphocyte proliferation and for inhibition of Mv-1-Lu cell proliferation.

Testing of fractionated HPVIII batch 30540 in lymphocyte proliferation assays revealed four peaks of lymphocyte inhibitory activity, peaks A, B, C and D, Figure 50. The first with an elution volume of 95 ml (**Peak A**. Molecular weight >970 kDa), a second minor peak at elution volume 144 ml (**Peak B**. Molecular weight approximately 550 kDa), a third at elution volume 210 ml (**Peak C**. Molecular weight <68 kDa) and a fourth at 272 ml (**Peak D**. Molecular weight <68 kDa). PBS, used as elution buffer had no effect on lymphocyte proliferation.

Testing for active TGF-β in a TGF-β bioassay revealed the presence of three inhibitory peaks, Figure 52; the first at elution volume 192 ml (**Peak E**), the second at elution volume 215 ml (**Peak F**), the third with an elution volume of 300 ml (**Peak G**), all with a molecular weight <68 kDa.

From this experiment it can be concluded that when HPVIII batch 30540 is fractionated, four peaks of lymphocyte inhibitory activity are revealed, with molecular weights >970 kDa, ~550 kDa, and two peaks with a molecular weight <68 kDa. The two peaks with a molecular weight <68 kDa also inhibiting Mv-1-Lu proliferation as well as lymphocyte proliferation, suggesting that these fractions contain active TGF-β or some activity which inhibits Mv-1-Lu proliferation.

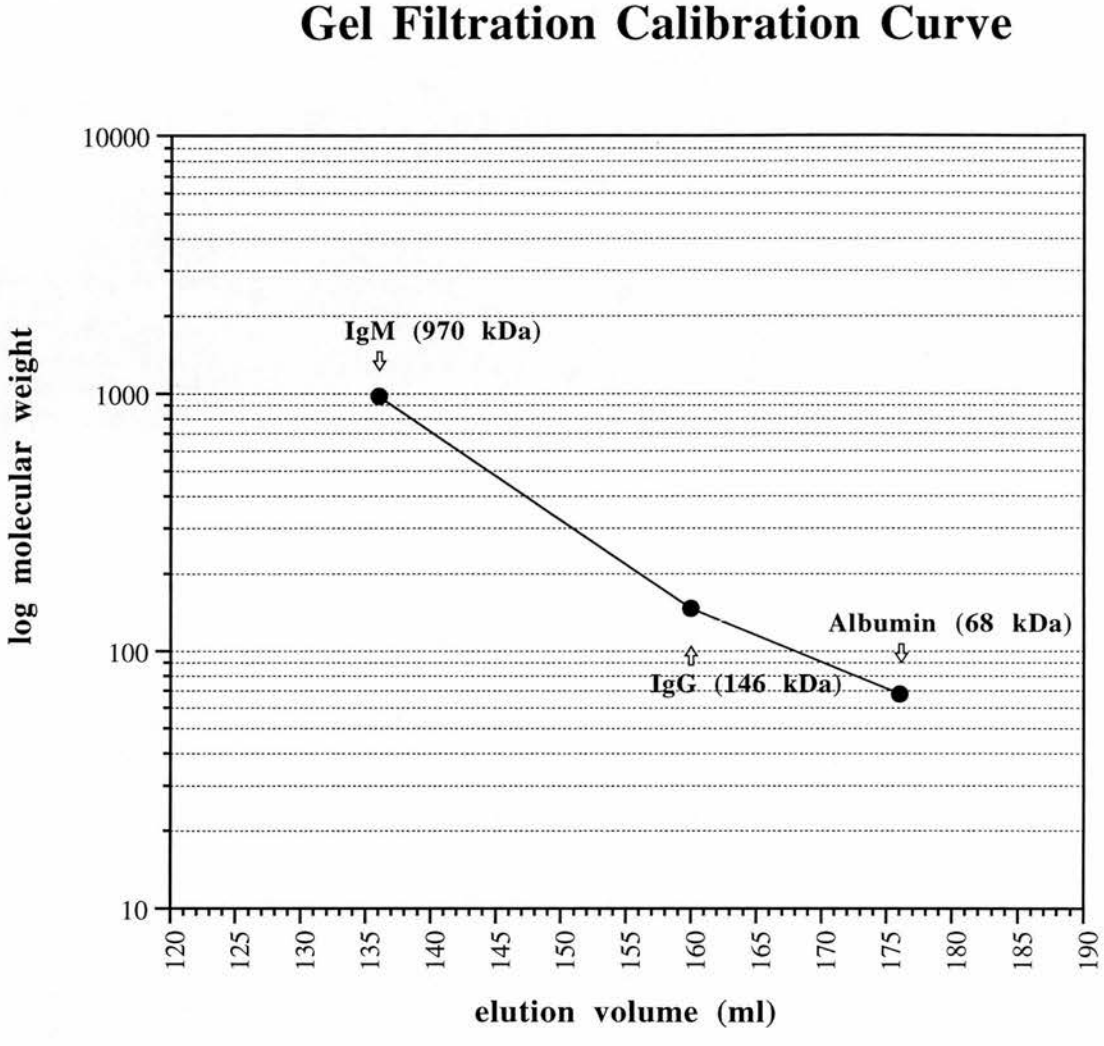


FIGURE 49: Calibration curve for column 1, Sepharose CL 4B, useful fractionation range for globular proteins 60-20,000 kDa. The column was calibrated by monitoring the elution volumes of proteins from human serum.

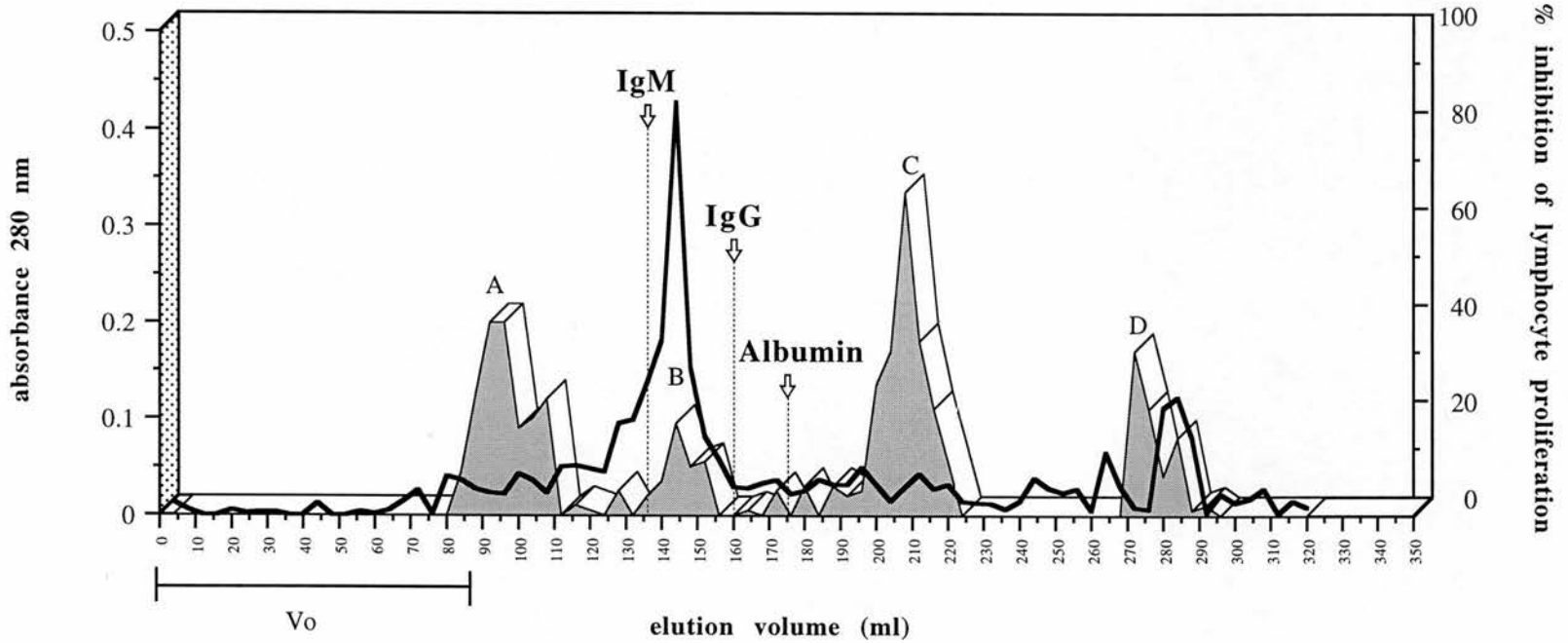


FIGURE 50: Elution profile of HPVIII (batch 30540) on Sepharose CL 4B (useful fractionation range 60-20,000 kDa). Fractions were assayed for protein by absorbance at 280 nm and for their ability to inhibit PHA-stimualted lymphocyte proliferation. Eluent = PBS. Sample volume = 4 ml. Void volume = 84 ml.

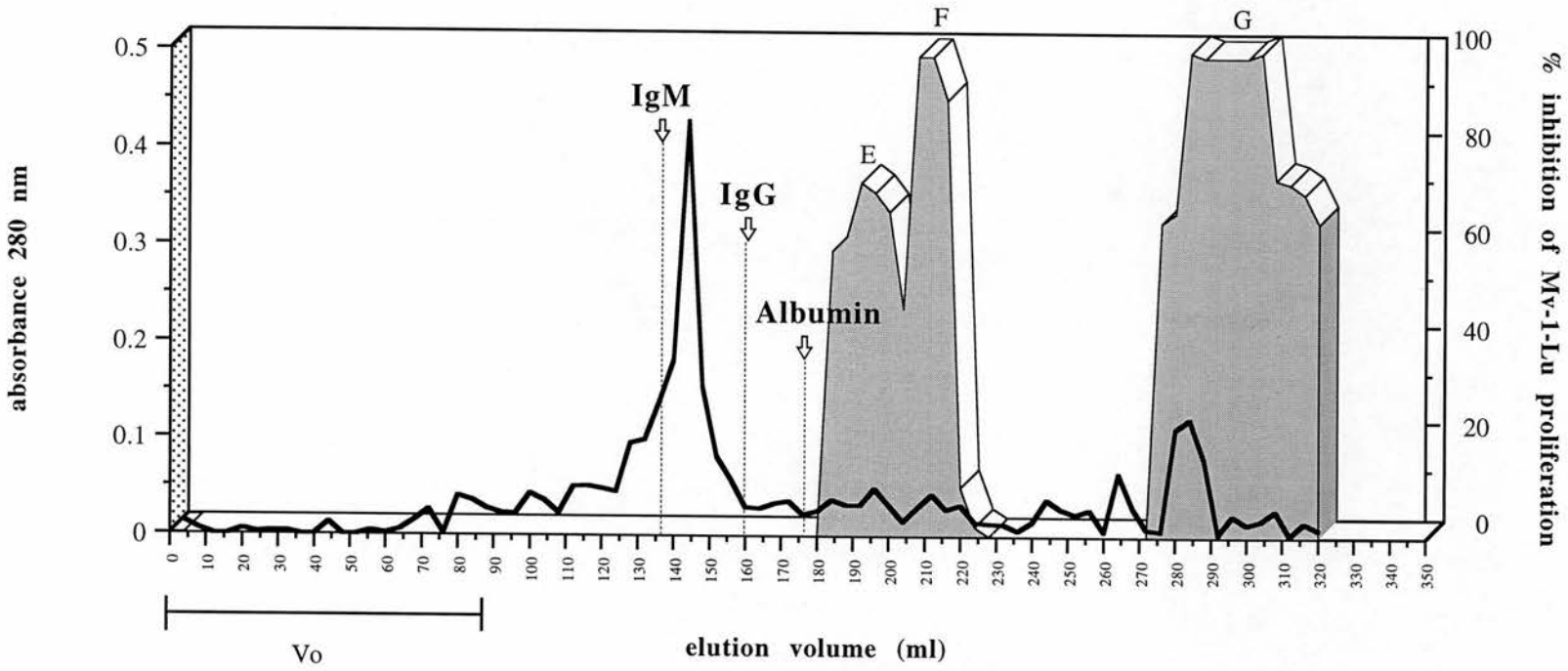


FIGURE 51: Elution profile of HPVIII (batch 30540) on Sepharose CL 4B (useful fractionation range 60-20,000 kDa). Fractions were assayed for protein by absorbance at 280 nm and for their ability to inhibit Mv-1-Lu cell proliferation. Eluent = PBS. Sample volume = 4 ml. Void volume = 84 ml.

absorbance

% inhibition of Mv-1-Lu cell proliferation by gel filtration fractions of HPVIII

(ii) Fractionation of HPVIII through Column 2.

Column 2 was packed with the gel filtration media Sephadex G-150, which has a fractionation range of 5-300 kDa for globular proteins. As with column 1, the Vo was determined, and the column calibrated before fractionation of the test sample. The Vo was found to be 45 ml. A calibration curve of log molecular weight of standards versus elution volume for column, is shown in Figure 52. A total of 315 IU/ml (5 mg of protein) of HPVIII 30650 was applied to the column and 200 fractions of a 1 ml volume collected.

Two protein peaks were detected by absorbance at 280 nm, Figure 53. The first was observed at elution volume 52 ml, and a second minor peak at 60 ml.

Testing fractionated HPVIII batch 30650 in lymphocyte proliferation assays revealed four peaks of inhibitory activity, Figure 53. The first with an elution volume of 52 ml (**Peak 1**. Molecular weight >970 kDa), which corresponds to the major protein peak. A second minor peak was observed at elution volume 62 ml (**Peak 2**. Molecular weight ~220 kDa), corresponding to the minor protein peak. A third peak occurred at elution volume 69 ml (**Peak 3**. Molecular weight ~120 kDa) and the fourth at 160 ml (**Peak 4**. Molecular weight ~6.5 kDa).

To determine if this fourth low molecular weight peak was due to low molecular weight stabiliser, such as citrate, which is used in the formulation of this product, a sample of formulation buffer containing these stabilisers was applied to column 2, Figure 54. The buffer was found to elute at 150 ml, which is approximately the same point as the low molecular weight components of HPVIII.

From fractionation of HPVIII batch 30560, it can be concluded that this batch of concentrate also contains four peaks of inhibitory activity, with molecular weights >970 kDa, ~220 kDa, ~120 kDa and ~6.5 kDa.

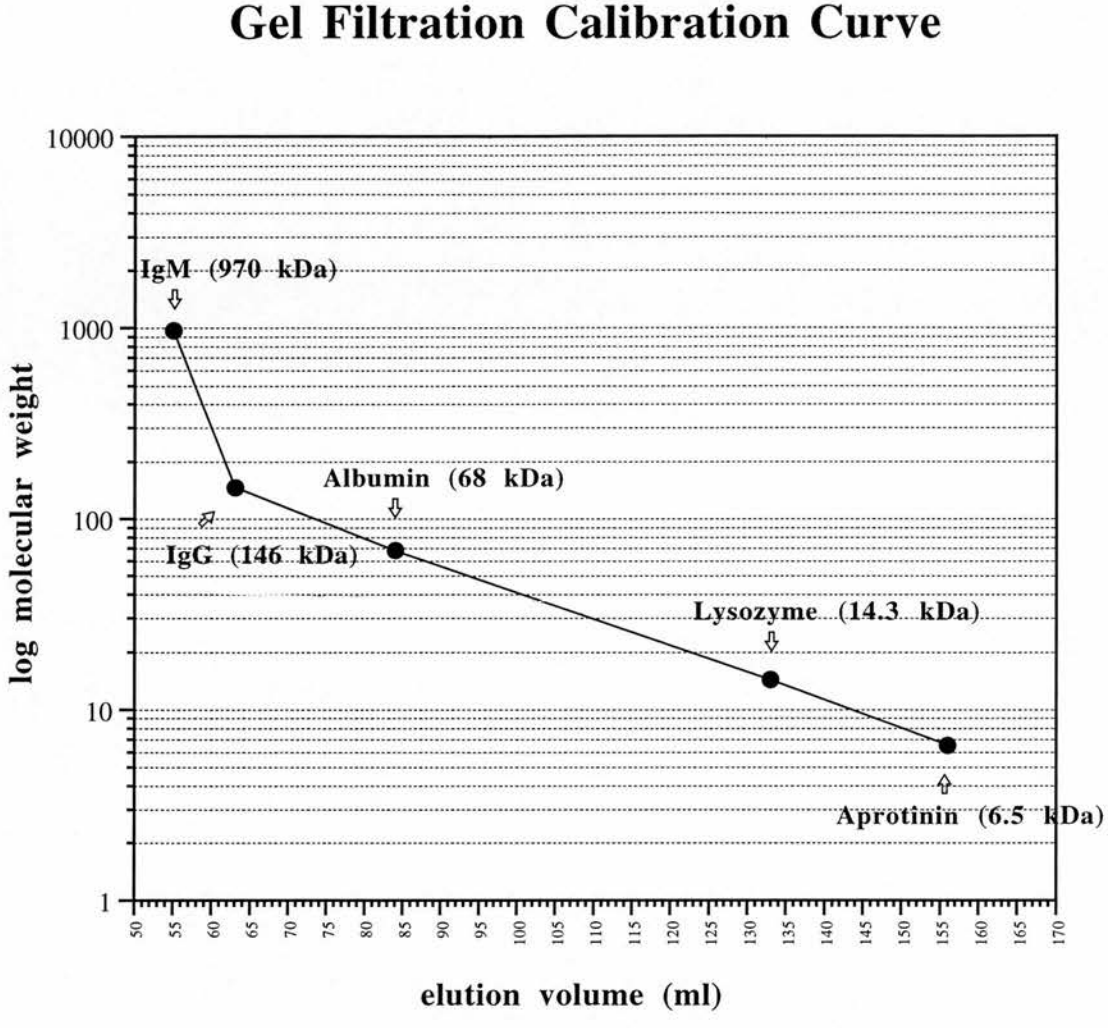


FIGURE 52: Calibration curve for column 2, Sephadex G-150, useful fractionation range for globular proteins 5-300 kDa. The column was calibrated by monitoring the elution volumes of proteins in human serum and purified protiens applied to the column.

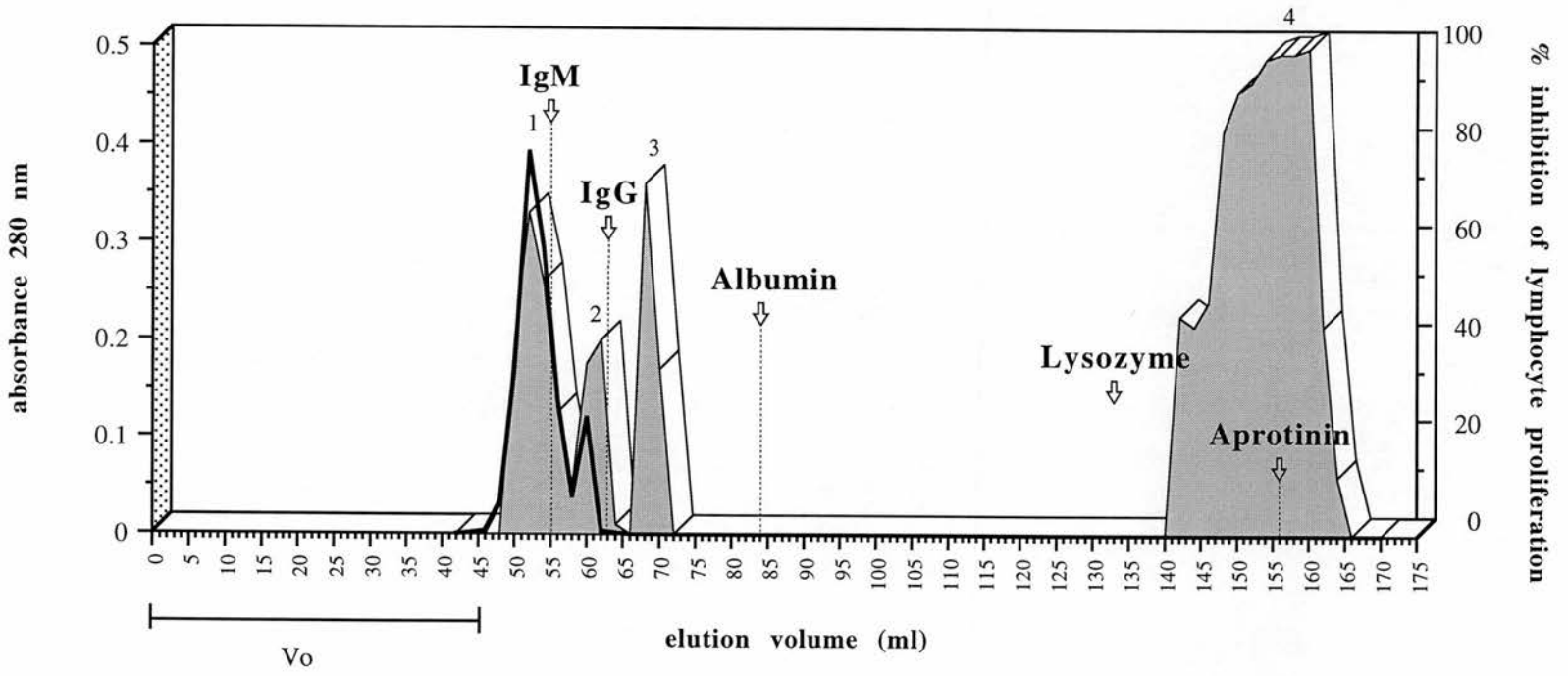


FIGURE 53: Elution profile of HPVIII (batch 30650) on Sepadex G-150 (useful fractionation range 5 -300 kDa). Fractions were assayed for proteins by absorbance at 280 nm and for their ability to inhibit PHA-stimulated lymphocyte proliferation. Eluent = PBS. Void volume = 45 ml.

——— absorbance

▒ % inhibition of PHA-stimulated lymphocyte proliferation by gel filtration fractions of HPVIII

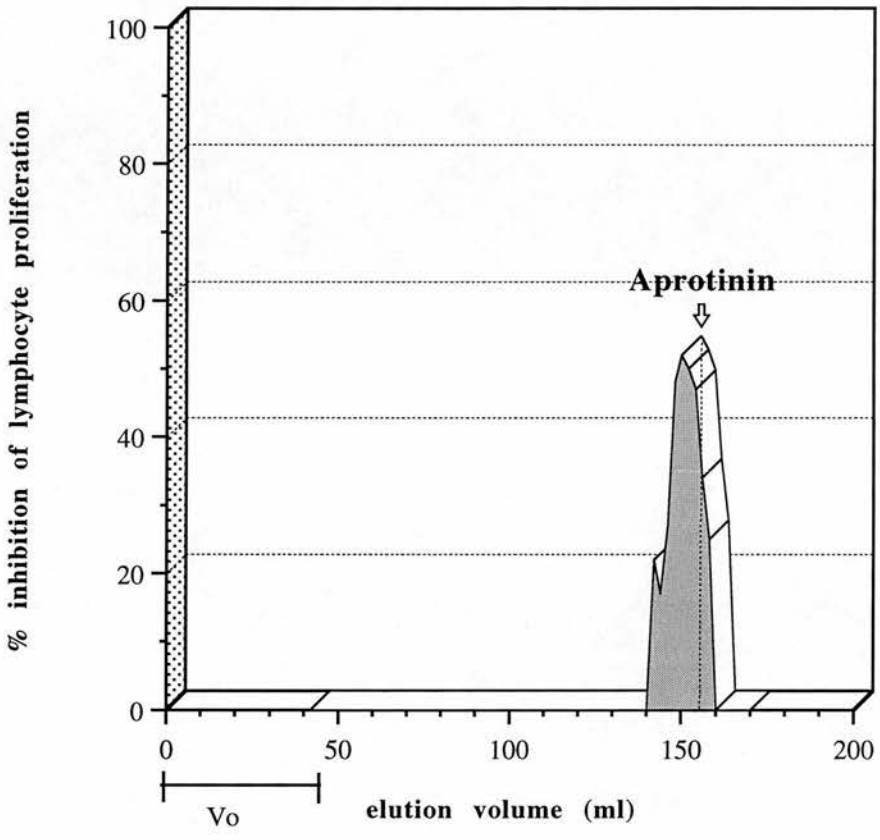


FIGURE 54: Elution profile of HPVIII formulation buffer on Sephadex G-150 (useful fractionation range 5-300 kDa). Fractions were assayed for their ability to inhibit PHA-stimulated lymphocyte proliferation. Eluent = PBS. Void volume 45 ml.

2. Testing of Other Cytokines in Lymphocyte Proliferation Assays

The cytokines IFN α, IFN γ and TNF α, were tested for their effects on PHA-stimulated lymphocyte proliferation. Both IFN α and TNF α inhibited lymphocyte proliferation to some extent, Figure 56. The inhibition of proliferation caused by TNF was not significant. IFN γ enhanced the proliferation of PHA-stimulated lymphocytes.

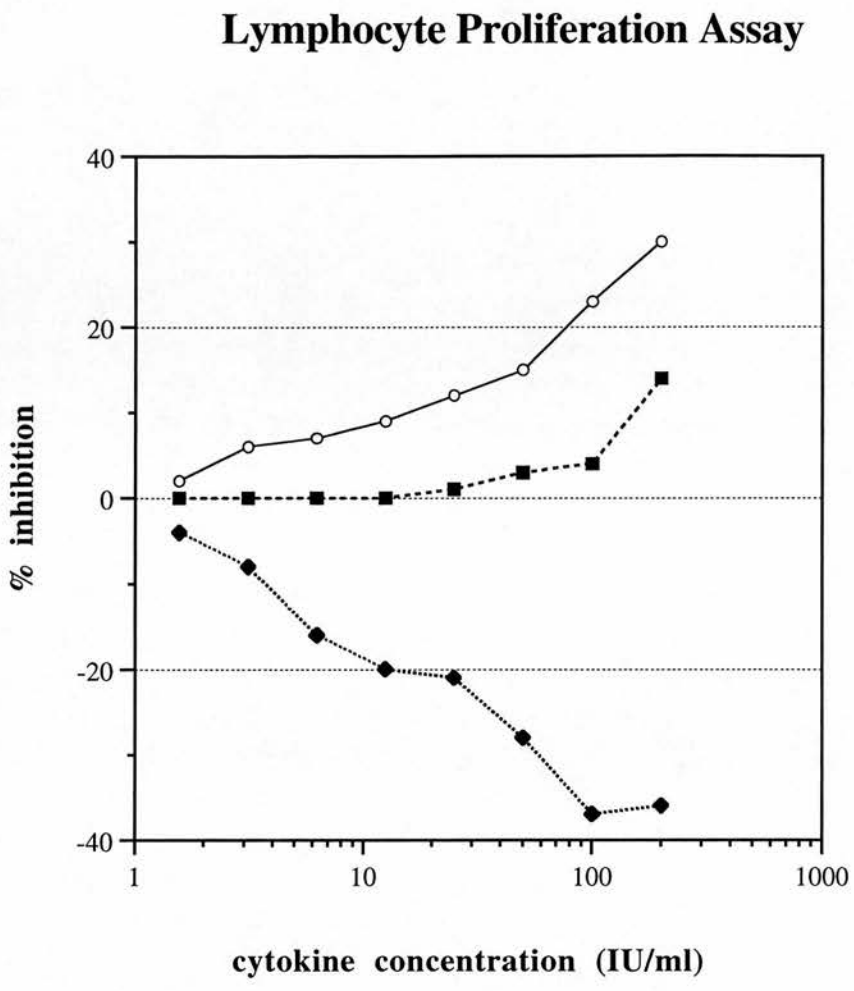


FIGURE 55: Effect that various cytokines have on PHA-stimulated lymphocyte proliferation.

IFN α

IFN γ

TNF α

DISCUSSION

1. Identification and Characterisation of Other Protein Components in Coagulation Factor Concentrates

The presence of other proteins in coagulation factor concentrates, besides the coagulation factor itself, was determined by electrophoretic separation and visualisation of proteins by silver staining. Protein components of factor VIII concentrates of varying purity were separated and visualised by this method. To achieve effective separation over a wide range of molecular weights, concentrates were separated through four formulations of polyacrylamide gel. Many protein bands other than those attributable to factor VIII or human albumin were detected. The only band identified whose appearance correlated precisely with lymphocyte inhibitory activity in the concentrates, was one with a molecular weight >200 kDa. Biologically active TGF-β homodimer or monomers of TGF-β could not be identified conclusively by this method, perhaps due to the minute amounts (0.02 ng TGF-β) on the gels being below the limit of detection of this system.

Having demonstrated that proteins other than the coagulation factor itself are present in coagulation factor concentrates, an inhibitory concentrate was treated with trypsin. This crude method was hoped to be able to determine whether all the inhibitory activity was due to protein contamination. Approximately 34% inhibition of lymphocyte proliferation was removed by treatment with 0.25 mg/ml trypsin:trypsin inhibitor complex, higher concentrations of the trypsin:trypsin inhibitor complex inhibiting lymphocyte proliferation. However, if the inhibition caused by high levels of the complex were to be subtracted from the inhibition due to trypsin-treated Profilate OSD, approximately 59% of the inhibitory activity of 2 IU/ml Profilate OSD could be removed by the highest concentration of trypsin, suggesting that 59% of the activity of this concentrate is due to protein the other 41% being trypsin -resistant protein or non-protein.

1.2. Dialysis of Coagulation Factor Concentrates

Several studies have shown that the inhibitory activity of factor VIII concentrates on lymphocyte function is removed, completely or partially, following dialysis. Since, a portion of the inhibitory activity of Profilate OSD was trypsin resistant, this concentrate was dialysed to establish whether any portion of the remaining activity was due to some low molecular weight component. No portion of this concentrates inhibitory activity on PHA-stimulated lymphocyte proliferation was removed by dialysis. This is in agreement with data obtained by Wadhwa *et al* (1992) using the same concentrate.

Other concentrates of varying purity were also dialysed. Approximately 40% of the inhibition of PHA-stimulated lymphocyte proliferation by two ion-exchange purified factor VIII concentrates was abrogated by dialysis, whereas only 25% of the affinity purified factor VIII concentrates' activity was removed. These results suggest that a dialysable component with a molecular weight < 2 kDa, present in ion-exchange and affinity purified concentrates, is responsible for a portion of the inhibitory activity of these concentrates. These results are similar to those obtained by Wadhwa *et al*, who on dialysing factor VIII concentrates, reported that a greater portion of ion-exchange concentrates' inhibition of IL-2 secretion was dialysable than that of intermediate purity concentrates. The dialysable components of ion-exchange concentrates are most probably the stabilisers used in the manufacturing processes, such as sodium citrate and amino acids, used to increase the ionic strength of the preparations and as bulking agent.

This technique is quite unrefined, and difficult to adequately control, since many factors may change in the overnight dialysis such as solution pH. In addition, components other than those < 2 kDa may be lost through unwanted adherence to the daialysis tubing. Accordingly no firm conclusions can be made from the data obtained. Nontheless, even thought the dialysis protocols used by myself and Wadhwa *et al* vary, the dialysable percentage of inhibitory activity obtained for identical concentrates is very similar, perhaps adding credence to these data.

1.3. Fractionation of Coagulation Factor Concentrates

Fractionation of two batches of the ion-exchange purified factor VIII concentrate through two different gel filtration media revealed the presence of many peaks of inhibitory activity. Gel filtration of HPVIII batch 30540 produced four peaks of inhibitory activity, one of high molecular weight, which came off the column straight after the void volume, the second a minor peak which corresponded to the protein peak, and two peaks with a molecular weight <68 kDa which inhibited both lymphocyte proliferation and Mv-1-Lu cell proliferation, suggesting that they contain active TGF-β. In conclusion, the lymphocyte inhibitory activity of this batch of concentrate seems mainly to be composed of an unidentified high molecular weight component >970 kDa and active TGF-β. The small inhibitory peak, with a molecular weight of approximately 550 kDa, is probably due to the effect of protein concentration in these fractions.

Gel filtration of HPVIII batch 30650 through Sephadex G-150, which has a lower molecular weight fractionation range than the Sepharose CL 4B used to fractionate batch 30540, also revealed the presence of four peaks of inhibitory activity. The first peak with a molecular weight corresponding to the major protein peak, the second corresponding to the minor protein peak and a third with a molecular weight of approximately 120 kDa. A fourth peak was observed which was subsequently shown to be formulation buffer. No peak was observed at the fractions corresponding to the correct molecular weight for active TGF-β, perhaps due to this batch of concentrate containing a smaller amount than the batch fractionated on Sepharose CL 4B. In conclusion, the main activity in this batch of HPVIII appears to be due to three components, two unidentified with molecular weights >970 kDa and 120 kDa and a third component which is most probably formulation buffer. Thus, by fractionating two batches of this concentrate using two types of gel filtration media, I have demonstrated the presence of the following lymphocyte inhibitory activities.

- >970 kDa unidentified
- ~550 kDa protein, unidentified
- ~220 kDa protein, unidentified
- ~120 kDa unidentified
- <68 kDa contains active TGF-β
- <6.5 kDa formulation buffer

Wadhwa *et al* (1994) also performed gel filtration experiments in order to determine the molecular weight of contaminants. The concentrate chosen for their experiments was an intermediate purity factor VIII concentrate. Fractionation revealed the presence of two peaks if inhibitory activity at 60 kDa and 200 kDa. Allowing for differences in column media, dimensions and slight calibration discrepancies, it would appear that the peak I have observed at < 68 kDa correspond to that observed by Wadhwa *et al.* It is suggested by the authors that the two peaks of inhibition are due to contamination with TGF-β, confirming this by neutralisation experiments. By testing the fractions in a TGF-β bioassay my results agree that the < 68 kDa fraction (their data 60 kDa) is indeed due to TGF-β. However, close examination of the neutralisation data for these fractions shows only a portion of the activity being neutralised by antibody to TGF-β.

In the concentrate I have chosen for fractionation, an ion-exchange purified factor VIII concentrate, I have detected five other peaks of inhibitory activity, > 970 kDa, ~550 kDa, ~220 kDa, ~120 kDa and <6.5 kDa. Only the < 6.5 kDa peak being identified as formulation buffer. The reason for these other peaks not being detected by Wadhwa *et al* may be due to the use of different concentrates. However, one would expect the ion-exchange to contain less contaminants. More likely the difference is due to the assay system used to detect immunomodulatory activity. As previously discussed the PHA-stimulated lymphocyte proliferation assay may be inhibited by more than just one mechanism, as well as by inhibition of IL-2 secretion.

CHAPTER 6

General Discussion

1. Effects of Coagulation Factor Concentrates on Normal Lymphocytes.

I have performed a comprehensive study on the effects that a range of coagulation factor concentrates of varying purity have on lymphocyte function *in vitro*. A general trend emerged throughout the data whereby, as product purity increased the inhibition of lymphocyte function *in vitro* decreased. Many other studies have focused on factor VIII concentrates alone. In this study factor IX and von Willebrand factor concentrates were also included. The results demonstrated that coagulation factor concentrates other than factor VIII concentrates are just as inhibitory to lymphocyte function. Where appropriate, the data obtained from this study was compared to that published by others, and found to be in close agreement.

The relevance of these *in vitro* studies to the immune modulation demonstrated by haemophiliacs receiving coagulation factor therapy remains unclear. I have shown that factor VIII and factor IX concentrates of the same purity group are as inhibitory as each other *in vitro*. However, this does not seem to be reflected in the *in vivo* data, as researchers have found that while haemophilia A patients demonstrate immune abnormalities, haemophilia B patients displayed immune characteristics similar to that of negative controls (Carr *et al*, 1984; Cuthbert *et al*, 1992). This may be explained by the presence of different inhibitory contaminants in factor VIII, factor IX and von Willebrand factor concentrates. It is not unlikely that the different techniques used to manufacture these products gives rise to different contaminants or abundance of those contaminants. Those present in factor VIII concentrates inhibiting lymphocyte function both *in vitro* and *in vivo*, whereas, those present in other coagulation factor concentrates only inhibiting lymphocyte function *in vitro*. Thereby, resulting in divergent results between haemophilia A and B patients. Nonetheless, this suggests that what is observed *in vitro* cannot necessarily be translated to the *in vivo* situation. Therefore, one must be cautious that conclusions derived from *in vitro* data as to the immunomodulatory effects of products is not applied to the *in vivo* situation.

2. Effects of Coagulation Factor Concentrates on the Immune System *in vivo*

In vitro studies for assaying the immune modulating capacity of coagulation factor concentrates are useful, however, they do not give us any information on the impact that concentrates may have on the immune system *in vivo*. The only way to assess this is to compare one concentrate to another in terms of variation in some immune parameter, such as $CD4^+$ count.

To date there are few studies on HIV negative haemophiliacs comparing the effects of different purity products on $CD4^+$ counts. The data available suggest that $CD4^+$ counts do decline in haemophiliac patients albeit at a slower rate than in HIV infected patients, and this decline is observed regardless of whether they are receiving intermediate or high purity products (Teitel *et al*, 1989; Cuthbert *et al*, 1990; Fukatake *et al*, 1991). Nevertheless, there is debate as to whether this apparent decline in $CD4^+$ counts is significant. Furthermore, there has been a study on previously untreated patients which found that $CD4^+$ counts remain within the normal range, regardless of product purity (Evans *et al*, 1991).

The data from comparison studies on HIV positive haemophiliacs are also contradictory. Several studies suggest that treatment with higher purity products stabilises $CD4^+$ counts (Brettler *et al*, 1989; de Biasi *et al*, 1991; Seremitis *et al*, 1990; Goldsmith *et al*, 1991; Seremitis *et al*, 1993; Goedert *et al*, 1994). These results are highly controversial since, if higher purity concentrates are capable of stabilising $CD4^+$ counts in HIV positive haemophiliacs, it could be reasoned that replacement therapy may be of benefit to individuals who are not haemophiliacs but HIV positive! Concern has also been expressed about the design of some areas of these studies. Much of the impact that they have is weakened by the fact that retrospective controls were used, that the $CD4^+$ counts in the test and control groups was often not equivalent and that the assignment of patients to purity groups was not random. In summary, studies of this nature to date are inconclusive. It therefore remains the subject of intensive debate as to whether there is any benefit from treatment with higher purity products. Until such comparison studies have been completed successfully no firm conclusions can be drawn regarding the immune modulating effects of products from one particular purity group.

Nonetheless, there is no reason to believe that higher purity concentrates would be more harmful than less purified concentrates. If higher purity concentrates were to extend the life span of HIV positive patients, can clinicians afford to wait until data from randomised trails are available? In my view a move towards increasing use of immunoaffinity purified or recombinant coagulation factor concentrates will finally eliminate anxieties about clinically relevant immune modulation.

3. Contribution of Blood-Borne Virus Infection to the Immune Abnormalities Observed in Haemophiliacs

The extent to which coagulation factor concentrates contribute to the immune modulation observed in haemophiliacs is also under debate. Two recent studies suggest that chronic blood-borne virus infection is a major contributory factor to abnormalities observed *in vitro* in cells of the immune system derived from haemophiliac boys and that factor VIII concentrates play a minor role (Evans *et al*, 1995; Pasi *et al*, 1995).

The proliferative response of T lymphocytes, derived from groups of haemophiliac boys to the lectins PHA and Con A were assessed. The proliferative response of T lymphocytes to lectins was found to be normal in HCV and HIV uninfected boys, whereas the those infected with either HCV or both HCV and HIV have impaired responses (Evans *et al*, 1995). The impact of blood-borne virus infection on monocyte antigen presentation and cytokine secretion was also investigated in the same groups of boys. A reduction in cytokine production was observed only in boys infected with both HIV and HCV, suggesting that HIV as an aetiological agent. Whereas, antigen presentation was reduced in boys infected with only HCV or both HCV and HIV (Pasi *et al*, 1995). The authors conclude that this evidence suggests that the impairment in the immune response observed in haemophiliac cells in culture is due to infection with blood borne viruses such as hepatitis C and HIV.

These findings do not entirely agree with *in vivo* data published by other researchers (Carr *et al*, 1984; Cuthbert *et al*, 1992). If viral infection was the cause of immune modulation in haemophiliacs, why are immune changes so mild in haemophilia B

patients? Infection with blood-borne viruses also does not explain the inhibitory effect that coagulation factor concentrates have on the PHA-stimulated proliferation of normal donor lymphocytes, observed by myself and many other researchers. Evans *et al* (1995) suggest that the concentrations of coagulation factor used in these studies have been variable and often substantially greater than would be achieved *in vivo* following treatment. I have found that with many coagulation factor concentrates significant inhibition of PHA-stimulated lymphocyte transformation occurs at concentrations as low as 0.1 IU/ml, which are indeed readily achievable *in vivo* following treatment.

This issue may be resolved through longer term studies such as the continued monitoring of the virally uninfected boys for clinically relevant immune abnormalities. However, as suggested before the move towards treatment with recombinant purified products would hopefully finally eliminate anxieties about immune modulation. and whether the cause is viral or due to contaminants in concentrates.

4. Is TGF-β Involved in Factor VIII Induced Suppression of Immune Function?

TGF-β comprises a family of multifunctional polypeptides that regulate the cellular growth and differentiation of a wide variety of cells (Sporn *et al*, 1987; Massagué, 1990). The TGF-β family in man consists of TGF-β1, TGF-β2 and TGF-β3. TGF-β is released from cells, and circulates in plasma as a latent complex which does not bind to receptors (Pricher *et al*, 1986; Wakefield *et al*, 1988; Massagué, 1990). Several configurations of latent TGF-β have been identified, including the large latent complex and the TGF-β-α_2M) form (O'Connor-McCourt & Wakefield, 1987; Wakefield *et al*, 1988; Huang *et al*, 1988).

The large latent complex, secreted by many cell types including platelets, lymphocytes and macrophages, is comprised of two distinct gene products with a total molecular weight of 235 kDa. It consists of the mature TGF-β (a 25 kDa disulphide linked homodimer), a second 75 kDa dimer, the latency associated

protein (LAP) and a 135 kDa latent TGF-β binding protein (LTBP) (Wakefield *et al*, 1988). LAP is linked by a disulphide bond to the LTBP, which is the product of a separate gene while the TGF-β dimer is associated with the LAP by electrostatic interactions (Sha *et al*, 1991). Following secretion of the latent form of TGF-β, activation must occur to release the biologically active dimer form. Latent TGF-β can be activated by a variety of chemical and physical treatments, including extremes of pH or temperature, detergents, proteases or urea (Lawrence *et al*, 1985; Miyazono *et al*, 1990; Brown *et al*, 1990), suggesting that latency is maintained by non-covalent forces.

It has been demonstrated by immunoassay that all detectable TGF-β in plasma and serum is bound to the 720 kDa glycoprotein α_2M, implying that α_2M is the major carrier of active TGF-β (Huang *et al*, 1987; O'Connor-McCourt & Wakefield, 1987). Dissociation of TGF-β-α_2M complex can be achieved under mildly acidic conditions (Huang *et al*, 1988; O'Connor-McCourt & Wakefield, 1987). Heparin and dextran sulphate are also capable of dissociating TGF-β form α_2M (LaMarre *et al*, 1990; McCafferty *et al*, 1989) suggesting that proteoglycans on the cell surface may dissociate TGF-β-α_2M complexes similarly.

The starting material for all coagulation factor concentrates used in my study, except those manufactured by recombinant techniques, is human plasma. Since TGF-β is derived from platelets, lymphocytes and macrophages, it is probably not surprising that plasma derived concentrates contain TGF-β. Prior to the coagulation factor fractionation process donated plasma is frozen until processing and melted slowly to produce cryoprecipitate. As postulated by Wadhwa *et al* (1994), this treatment of the plasma may cause the lysis of residual platelets contained in the plasma and hence the release of latent TGF-β into the feedstock. Latent TGF-β, as previously discussed, can be activated *in vitro* by various physio-chemical treatments which include temperature and detergents. Since two of the most commonly used viral inactivation methods incorporated into the fractionation processes are solvent/detergent and heat treatment, it may be that latent contaminating TGF-β is being activated by these processes. TGF-β in plasma and serum is bound in a latent complex with α_2M, so in addition to latent TGF-β

released by platelet lysis, activation of biologically latent α_2M-TGF-β complex could contribute to the levels of active TGF-β detected in coagulation factor concentrates.

I have confirmed the findings of Wadhwa *et al* (1994) that most intermediate purity, ion-exchange and affinity purified factor VIII concentrates contained active TGF-β, whereas immunoaffinity and recombinant did not. In addition, I have also demonstrated that concentrates of other coagulation factors such as factor IX and von Willebrand factor also contain active TGF-β. Latent TGF-β1 was also found to present in all plasma derived concentrates. However, this appears to be remaining inactive throughout the culture period as even though immunoaffinity purified concentrates contain the latent cytokine they have no effect on lymphocyte proliferation.

Even though TGF-β is definitely a contaminant of coagulation factor concentrates the role that it plays in the *in vitro* inhibition of lymphocyte function appears to be minimal. The authors of the original report stated that the addition of specific TGF-β antibody reversed the inhibitory effect of some concentrates on IL-2 secretion. However, their neutralisation data is unconvincing, and clearly demonstrates that in most cases only a portion of the activity is due to TGF-β. Using many different approaches to neutralisation, including the method used by Wadhwa *et al* (1994), I found that the addition of excess antibody to TGF-β could not abrogate any of the inhibition of PHA-stimulated lymphocyte proliferation caused by concentrates. Only when a purified T lymphocyte system was used could a significant amount of the activity be removed by addition of antibody to TGF-β. The only conclusion that can be drawn from this is that the TGF-β present in concentrates is inhibiting a portion of T lymphocyte proliferation, most probably by inhibiting IL-2 secretion. However, the overall effect that TGF-β inhibition by this mechanism has on PHA-stimulated lymphocyte proliferation is minimal, since no reversal of the effects by a specific antibody was observed. This would agree with my previous conclusion that TGF-β levels serve purely as a marker of concentrate purity rather than being the sole and direct cause of inhibition of lymphocyte function *in vitro*.

Another factor to be considered is the impact that the levels of contaminating TGF-β contained in coagulation factor concentrates would have on the immune system of patients receiving therapy. As described in chapter 4, total TGF-β1 levels were measured in the plasma and serum of normal individuals, and the increase in the level of TGF-β1 calculated if that individual were to be transfused with a concentrate containing relatively high levels of contaminating TGF-β1. The results demonstrating that treatment would lead to an increase of only 0.5 ng/ml in total plasma TGF-β1, i.e. a change of only 1.3%.

It has been demonstrated that mice injected with TGF-β1 for the treatment of auto immune diseases have a marked depression in immunologic responses (Kuruvilla *et al*, 1991). However, these animals were given 2 μg/ml TGF-β, a far greater amount than that likely to be received by a haemophiliac receiving replacement therapy. In addition, pharmacokinetic studies in rats have shown that both active and latent TGF-β are cleared rapidly from the circulation, active TGF-β within a few minutes and latent TGF-β within 90 minutes (Wakefield *et al*, 1990). It seems inconceivable that the very small increase would have any impact on the immune system of an individual whose normal plasma TGF-β1 level is in the order of 41 ng/ml. Therefore, it may be reasonably concluded that TGF-β is not responsible for immune modulation observed in HIV-negative haemophiliacs. Furthermore, if TGF-β were to be responsible for the immune abnormalities observed in haemophiliacs *in vivo*, why then do haemophilia B patients and those with von Willebrands disease not demonstrate any clinically relevant immune abnormalities? Since, factor IX and von Willebrand factor concentrates both contain comparable amounts of TGF-β to factor VIII concentrates. This would again seem to suggest that TGF-β plays no role in observed immune disturbances.

5. Mechanism of Inhibition of PHA-Stimulated Lymphocyte Proliferation by Coagulation Factor Concentrates

Where the merit of using *in vitro* assays of immune modulation to determine whether a concentrate will be of harm to a patient is doubtful, they have been shown to be invaluable as a tool for screening for active contaminants in concentrates.

Many groups have attempted to elucidate the mechanism of inhibition of lymphocyte function by coagulation factor concentrates. The toxicity of intermediate purity factor VIII concentrates to PBMC has examined by McDonald *et al* (1985), Lederman *et al* (1986) and Wang *et al* (1986), by incubating PBMC with concentrate and counting viable cells, identified by trypan blue exclusion or cytofluorometery. Both groups reported that inhibition of lymphocyte proliferation by intermediate purity concentrates was not due to a mere cytotoxic effect. Vermot-Desroches *et al* (1992) confirmed the above observations using an ion-exchange product and assessing cell viability by Eosin Red dye exclusion. These observations have been confirmed by a two other groups (Hay *et al*, 1990; Wadhwa *et al*, 1992). No group had studied the effects of other inhibitory coagulation factor concentrates, or those purified by affinity techniques. Accordingly, I tested these preparations and found perhaps unsurprisingly that they did not mediate their inhibitory effects through cytotoxicity.

McDonald *et al* (1985) investigated whether the inhibition of PHA or Con A-stimulated proliferation was due to a direct interaction between the mitogens and some component(s) in intermediate purity factor VIII and factor IX concentrates. The mitogens were allowed to react in double diffusion plates against factor VIII of factor IX concentrates. No precipitation was detected between either clotting factor preparation and PHA. However, some precipitation was detected between ConA and both factor VIII and factor IX preparations. Perhaps more convincingly, simple binding of lectin by some component(s) in an intermediate factor VIII concentrate was also investigated by Lederman and colleagues (1986). Gel filtration of a mixture of iodine^{125}-labelled PHA and the factor VIII concentrate revealed no binding of isotope to fractions with inhibitory activity.

Direct binding of inhibitory component(s) in factor VIII concentrates to cell surface components involved in mitogen mediated triggering of IL-2 secretion was investigated by Wadhwa *et al* (1992). Attempts were made to adsorb substance(s) of this nature by incubation of factor VIII concentrates with resting or PHA-activated T cells. No reduction in inhibitory activity in those concentrates absorbed with activated or non-activated cells was observed. These results imply that inhibitory component(s) mediate their effect by mechanism(s) which do not involve simple blocking of cell surface components involved in mitogen stimulation.

The time course of the effect of an ion-exchange purified factor VIII concentrate on PHA-stimulated lymphocyte proliferation has also been investigated by adding preparations at different times after the activation of proliferation by the mitogen (Vermot-Desroches *et al*, 1992). The results showed that maximum inhibition was obtained when the concentrate was added 6 hours after PHA addition. When the concentrate was added after this time, the degree of inhibition was gradually reduced, suggesting that the inhibitory influence of this product was limited to an early event in the process of lymphocyte activation. Similar results were obtained by Wang *et al* (1986) who observed that when intermediate purity factor VIII concentrate was added later than 8 hours after the stimulant, the degree of inhibition was reduced. However, the maximum inhibition was observed when concentrate was added simultaneously with mitogen.

The kinetics of inhibition of IL-2 secretion by an intermediate purity factor VIII concentrate was also investigated by Wadhwa *et al* (1992). Their results demonstrated that delayed exposure of a T cell line to the factor VIII concentrate for more than 2 hours resulted in the progressive attenuation of the inhibitory effect on PHA induced IL-2 secretion.

The inhibition of lymphocyte function by some factor VIII concentrates is thought to be independent of monocyte interaction (Hay *et al*, 1990; Vermot-Desroches *et al*, 1992). Hay and colleagues found that lymphocyte proliferation in response to phorbol-myristate-acetate (PMA), a reaction which is not reliant on monocyte interactions, was inhibited by some factor VIII concentrates. Furthermore, the

depletion of monocytes from PBMC preparations to < 1% did not affect some factor VIII concentrates' ability to inhibit lymphocyte proliferation.

The role of monocytes in the inhibition of lymphocyte mitogenic functions caused by an ion-exchange purified factor VIII concentrate was also investigated by Vermot-Desroches *et al* (1992). As pure T lymphocytes do not proliferate in response to mitogen, monocyte interaction was tested by measuring T lymphocyte proliferation in the presence of varying numbers of monocytes. A similar degree of inhibition of PHA-stimulated lymphocyte proliferation was observed, irrespective of the percentage of monocyte cells.

In summary, these data from several research groups indicate that the inhibitory effect of factor VIII concentrates is not exerted through a simple blocking of cell surface components, binding of mitogen or direct cytotoxicity. Several independent reports also conclude that the inhibitory component exerts its effect almost immediately on addition of the concentrate. Having reviewed this data, I concluded that it was probably unnecessary to study this further, but it would be more constructive to focus on the identification and characterisation of inhibitory components in concentrates.

6. Identification and Characterisation of Other Components in Coagulation Factor Concentrates

One must postulate that if TGF-β is capable of evading the fractionation process to be found in active form in coagulation factor concentrates, that many other cytokines or biologically active substances may also contaminate these concentrates. Some of them perhaps having an impact on *in vitro* immune function. Accordingly further investigation into the possible nature and number of these contaminants was necessary.

Many factors have been considered to be causing immune abnormalities in haemophiliacs. It has been proposed that the *in vitro* effects of coagulation factor concentrates are due to some specific component(s), rather than being a "non-specific" function of protein concentration. Wadhwa *et al* (1992) reported that the inhibition of IL-2 secretion from T cells by factor VIII concentrates differed considerably among the various intermediate purity concentrates, suggesting that the inhibitory action was due to some specific component(s). My data also supports this view, since within purification categories there was some variation in the effects concentrates have on both assays.

The coagulation factors themselves have also been implicated as being immunomodulatory. However, it is unlikely that factor VIII or factor IX are responsible for the previously mentioned *in vitro* effects, since I have found that highly purified (immunoaffinity) factor VIII and factor IX products have no effect on PA-stimulated lymphocyte proliferation or IL-2 secretion. This is supported by the lack of effects that recombinant factor VIII products have on lymphocyte proliferation assays. This opinion is also upheld by other published data (Schreiber *et al*, 1987; Wadhwa *et al*, 1992; Wadhwa *et al*, 1994).

Albumin which is added as a stabiliser to some concentrates has also been implicated. However, the above results, which show a lack of inhibitory activity with immunoaffinity purified and recombinant products, also exclude albumin as being a component of coagulation factor concentrates causing these *in vitro* effects, since both immunoaffinity purified and recombinant factor VIII have human serum albumin added as a stabiliser. Furthermore, a purified preparation of human albumin

was tested by Thorpe *et al* (1989) for it's effects on IL-2 secretion by PHA-stimulated T cells and found to have no effect.

Other proteins present at considerable concentrations in intermediate purity factor VIII concentrates are fibronectin and fibrinogen. Schultz and Shahidi (1990 & 1990a) reported that fibronectin is a potent inhibitor of PHA-stimulated lymphocyte proliferation. The authors demonstrated that lymphocyte proliferation was inhibited in a dose-dependent manner over a range of concentrations of fibronectin (100-400 μg/ml). Similar levels of fibronectin are present when factor VIII concentrate are included in lymphocyte proliferation assays. In contrast, Wang *et al* (1986) reported that both purified fibronectin and fibrinogen had no effect on PHA-stimulated lymphocyte proliferation. It seems unlikely that fibrinogen or fibronectin are responsible, since, ion-exchange and affinity products some of which are very inhibitory to lymphocyte function contain only trace levels of these proteins.

It has been proposed that immune complexes or IgG aggregates are a possible cause of adverse immunological reactions. As previously mentioned, it has been demonstrated that both heat-aggregated IgG and certain factor VIII concentrates affect monocyte/macrophage function (Eibl *et al*, 1987; Mannhalter *et al*, 1988). The same group of investigators subsequently demonstrated that small amounts (ng/ml) of factor VIII-anti-factor VIII immune complexes could modulate Fc-receptor bearing cells (Mannhalter *et al*, 1990). However, as I have found that immunoaffinity purified factor VIII concentrates which contain trace amounts of these complexes (murine IgG complexed to factor VIII) do not affect PHA-stimulated lymphocyte proliferation or IL-2 secretion this does not seem likely. Again this view is supported by other researchers (Schreiber *et al*, 1987; Wadhwa *et al*, 1992). Furthermore, Thorpe *et al* (1989) has demonstrated that neither IgG preparations nor heat-aggregated IgG have any inhibitory effect on IL-2 secretion by PHA-stimulated T lymphocytes.

Recently the serum protein α_2-macroglobulin (α_2M) has been implicated as a contaminant in factor VIII concentrates. α_2M is one of the major plasma glycoproteins in man, its concentration in plasma varying between 2-4 mg/ml. In its native state, α_2M has a molecular weight of 720 kDa and consists of four identical polypeptide chain subunits of molecular weight 185 kDa which are held together by both disulphide and non-covalent bonds (James, 1980). The main biological function of α_2M has been attributed to its "pan-proteinase" binding activity, allowing it to interact with and capture virtually any proteinase (Barret & Starkey, 1973; Gonias *et al*, 1982). The proteinase inhibitory function of α_2M remains of uncertain physiological importance. α_2M also interacts with a wide variety of other molecules and ions, such as cytokines, immune complexes, lectins and other mitogenic substances, and a number of these reactions have been shown to impair immune responses (James, 1980). Cytokine binding by α_2M has been observed with platelet derived growth factor (PDGF), TGF-β, IL-2, IL-6, IFN-γ and tumour necrosis factor (TNF) (James *et al*, 1994). α_2M is thought to perform a carrier function in which cytokines circulate bound to α_2M, or a targeting role by which cytokines are delivered to cells with α_2M receptors.

Abstracts by Hay *et al* (1995 & 1995a) suggest that some factor VIII concentrates exert varying degrees of amidolytic activity which is related to α_2M enzyme complexes. Hay and colleagues tested a range of factor VIII concentrates for their amidolytic activity and their effect on lymphocyte function. A strong correlation between amidolytic activity and lymphocyte function was reported. Activity was found to be greatest in intermediate purity concentrates and barely detectable in monoclonally purified and recombinant products. Gel fractionation of concentrates revealed that molecular weight column fractions of 80,000 kDa of inhibitory concentrates exerted the most activity. The authors concluded that the reported inhibition of lymphocyte function *in vitro* may be due to the presence of α_2M and to a low molecular weight component.

In conclusion, for PHA-stimulated lymphocyte proliferation assays, the inhibitory activity caused by some coagulation factor concentrates is not due to the presence of the coagulation factor itself, albumin, fibronectin, fibrinogen or TGF-β. The contribution of α_2M to the inhibition of lymphocyte function observed *in vitro* at present remains unconfirmed.

I have fractionated two batches of an ion-exchange purified factor VIII concentrate using gel filtration, and have demonstrated the presence of six components which inhibit lymphocyte proliferation. Of the six peaks of inhibitory activity identified using this technique, only two of them were identified as containing a particular contaminant. One peak of inhibitory activity was found to be due to low molecular weight stabilisers which are used in the formulation of this product. However, whilst this activity causes measurable inhibition of lymphocyte proliferation *in vitro* it is highly unlikely that it will have any effect on lymphocyte function *in vivo*. Another peak with a molecular weight of < 68 kDa was found to contain active TGF-β. Whether it is responsible for the observed inhibition of lymphocyte proliferation remains undetermined, as unfortunately neutralising experiments using a specific antibody were not performed on these fractions. However, as discussed previously the amount of TGF-β present in this concentrate is unlikely to cause the immune abnormalities observed *in vivo*. Four other lymphocyte inhibitory activities with molecular weights > 970 kDa, ~550 kDa, ~220 kDa and ~120 kDa remain unidentified. The serum protein α_2M has been implicated as contributing to the inhibition of lymphocyte function observed *in vitro*. However, it seems unlikely that it contributes to the inhibition of lymphocyte proliferation by this concentrate, since no component of the corresponding molecular weight was revealed during gel filtration or SDS-PAGE. However, further experimentation would be required to confirm this interpretation.

These unidentified components which have been shown to inhibit lymphocyte function *in vitro*, may be the key to the immune abnormalities observed *in vivo* in haemophiliacs receiving these concentrates. If time were permitting, perhaps protein sequencing of these fractions might reveal the identity of some of these fractions allowing the relevance of contamination of concentrates with them to be

assessed. Furthermore, fractionation of inhibitory factor IX and von Willebrand concentrates may have revealed the presence of different contaminants to those present in factor VIII concentrates, perhaps explaining why haemophilia A patients display more immune abnormalities than haemophilia B or von Willebrand factor patients.

In summary, I have performed an extensive study on the effects of coagulation factor concentrates of varying purity on *in vitro* assays of immune function. I have made new observations regarding the effects of high purity concentrates on lymphocyte proliferation *in vitro* and have for the first time directly compared the effects of coagulation factor concentrates on both PHA-stimulated lymphocyte proliferation assays and lymphocyte IL-2 secretion. In addition, I have eliminated many of the possible candidates present in coagulation factor concentrates, reported to cause immune modulation *in vitro*. I have also demonstrated the presence of four previously unreported lymphocyte inhibitory activities present in an ion-exchange chromatography purified factor concentrate, however, further, investigation is required in order to identify these components.

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PUBLICATIONS RELATING TO THE WORK PRESENTED IN THIS THESIS

1. Speirs, H.J.L., Stirling, D., Ludlam, C.A. & Steel, C.M. (1995) TGF-β is only a minor immunosuppressive contaminant of factor VIII concentrates. *Thrombosis and Haemostasis*, **73,** 1036.
This work was presented at the International Society for Thrombosis and Haemostasis, 12-15th June 1995.

2. Speirs, H.J.L., Stirling, D., Ludlam, C.A. & Steel, C.M. (1996) A direct comparison of two *in vitro* assays of immune function caused by coagulation factor concentrates. *British Journal of Haematology*, **93,** 59.
This work was presented at the British Society for Haematology, 22-25 April 1996.

3. Speirs, H.J.L., Stirling, D., Ludlam, C.A. & Steel, C.M. (1996) Comparison of the effects of coagulation factor concentrates on two *in vitro* assays of immune modulation. *Blood Coagulation and Fibrinolysis*, **7,** 418-419.
This work was presented at the British Society for Haemostasis and Thrombosis, 1-3 May 1996.

Purification	Product	Batch Number	Coagulation Factor Activity (IU/ml)
Intermediate	Z8	1-369	10
	Z8	1-378	10
	Profilate SD	AR2003A	23
	Profilate SD	AR2004A	35
	Profilate SD	AR2008A	35
	Profilate OSD	AR4205A	49
	8Y	FHC 0484	25
	8Y	FHC 0494	25
	8Y	FHC 4133	26.5
	Haemate P	978641	26
	Defix*	20720	42.5
	9A*	FJA 0114	55
Mean			**30.2**
Range			**10 - 55**

Appendix 1(i):- Intermediate purity product coagulation factor activities.

Products marked with * are factor IX concentrates. All other concentrates are factor VIII concentrates.

Purification	Product	Batch Number	Coagulation FactorActivity (IU/ml)
Ion-exchange	HPVIII	10040	18
	HPVIII	20390	18
	HPVIII	30540	23
	HPVIII	30560	26.6
	HPVIII	30650	31.5
	HPVIII	40820	31.7
	Immunate	09H309210S	44.2
	Immunate	09H319210S	46
	Immunate	09H319301S	46
	Immunate	09H349211S	112
	Immunate	09H359212S	119
	Alpha VIII	AP2005A	108
	Alpha VIII	AP2006A	408
	Alpha VIII	AP2008A	108
	AlphaNine SD*	CA21401A	40
	AlphaNine SD*	CA2403AB	53
	Concentré De Facteur Willebrand#	87920070	27
Mean			**75.3**
Range			**18 - 403**

Appendix 1(ii):- Ion-exchange product coagulation factor activities.

Products marked with * are factor IX concentrates, those marked with # are von Willebrand factor concentrates. All other concentrates are factor VIII concentrates.

Purification	Product	Batch Number	Coagulation Factor Activity (IU/ml)
Affinity	Alphanate	AS4701A	98
	9MC*	PMC 2203	57
Mean			**77.5**
Immunoaffinity	8SM	FHD 4143	25
	Monoclate-P	J53706	25.5
	Mononine*	J82612	104
Mean			**63.1**
Recombinant	Kogenate	ADAH1	25
	Recombinate	95F22A250	100
Mean			**62.5**

Appendix 1(iii):- Affinity, immuoaffinity and recombinant product coagulation factor activities.

Products marked with * are factor IX concentrates. All other concentrates are factor VIII concentrates.

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